TRANSFECTED MESENCHYMAL STEM CELLS IN A THERMOREVERSIBLE HYDROGEL MATRIX FOR THE TREATMENT OF MYOCARDIAL INFARCTION

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

This research presented here was conducted for the purpose of finding an effective method of treating myocardial infarction utilizing genetically modified mesenchymal stem cells delivered in a thermoresponsive hydrogel. Heart disease is the most common cause of death in the industrialized world and treatment still has much room for improvement. Growth factor treatments have shown promise in revascularizing the infarcted tissue, provided either as a recombinant protein or by means of gene therapy. Stem cells are also a means of treating disease that is growing in effectiveness, although cells injected to the heart are often plagued by poor engraftment at the injection site.

This research is divided into three main sections. The first deals with the selection of an appropriate growth factor to be used in the study. Testing included quantifying the pro-angiogenic effect of the multiple growth factors on human endothelial cells as well as the growth factors' effects on the proliferation of the cells. Plasmid DNA constructs for the growth factors of interest were created for use in these studies. Growth factors were also tested for their influence on the mesenchymal stem cells. It was found that none of the growth factors or polymers used for DNA complexation and transfection resulted in differentiation of the stem cells. After completing this phase of the

research, platelet-derived growth factor “C” was chosen for use in the following phases of the work.

The second phase dealt with the characterization of a scaffold to be used with the transfected stem cells. After injection of cells in saline to the myocardium, 90% of the cells can be lost from the injection site. The hydrogel PoligoGel© from Samyang Corp. was found to be compatible with the stem cells, being both nontoxic, as well as not affecting differentiation of the cells.

The final portion of the research was the application of the growth factor, stem cells, and scaffold *in vivo* in a rat model to observe the effects of the treatment in the infarcted heart. Lewis rats underwent a coronary artery ligation to create an infarcted region of the heart. Stem cells transfected to express platelet-derived growth factor ‘C’ were then injected into the heart in a thermoreversible scaffold. The effectiveness of the treatment was quantified by analyzing MRI scans of the heart, as well as the biodistribution of the injected cells.

The biodistribution was performed by RT-PCR and showed a 15-fold increase in cells retained at the heart for groups injected with cells in a thermoreversible scaffold versus those injected in saline. The MRI data did not show any statistical significance between groups.

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CHAPTER 1

GENERAL INTRODUCTION, RATIONALE FOR THE STUDY, AND SPECIFIC AIMS

1.1 General Introduction

The potential of gene therapy and cell therapy is truly astounding. Multiple Nobel Prizes have been awarded for work in these fields(1); looking at just the past decade, we see it was awarded in 2008 "for the discovery and development of the green fluorescent protein, GFP," and in 2007 for "discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells" and "discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells." In 2006, it was awarded for the "discovery of RNA interference - gene silencing by double-stranded RNA." In 2002, "discoveries concerning genetic regulation of organ development and programmed cell death" earned the Prize for Medicine or Physiology. For 2001, it was awarded for "discoveries of key regulators of the cell cycle." There are also others from this time period with less direct

application in these fields, but still tangentially related, as well as many others awarded prior to 2000.

Gene therapy focuses generally focuses on one of two avenues; first is introducing a new gene construct to the cell to provide a new gene, or secondly to augment an existing gene. This has been used as a substitute for protein or peptide therapy and provides several advantages, including a longer therapeutic window as the half-life for the expression of the delivered gene is much higher than that of a protein, altered expression profile with different promoters, and potential for improved stability versus wild type protein by specific mutations in the protein (2). The method of action for gene therapy is the interruption of the expression of a gene accomplished by interfering nucleic acids (3).

The foreign genetic material can be introduced virally or nonvirally. Both methods have their limitations and advantages. Viruses have evolved specifically for the delivery of DNA/RNA, and are most effective at overcoming the physiological and cellular barriers to gene expression. They are, however, immunogenic (4). Nonviral methods, including polymers and liposomes, are nonimmunogenic, and can be administered repeatedly. The effectiveness of these methods still leaves much to be desired, as they are less effective at delivering the nucleic acids, especially to the nucleus (2, 5). The specific barriers that must be overcome prior to gene expression are degradation by both extra- and intracellular nucleases, clearance by the immune system, transport across the cell membrane, escape from the endosome, and transport

to and into the nucleus (6-8). These barriers are the same for gene silencing techniques with the exception of nuclear entry.

Cell therapy is also showing great potential in treating many diseases. Stem cells can generally be divided into one of three types: embryonic stem cells, adult stem cells, and induced pluripotent stem cells. Embryonic stem cells are capable of differentiation into any cell type in the body, given the right cellular signals, but suffer from some safety concerns, and much ethical controversy (9). Adult stem cells are obtained from mature tissues and do not require the destruction of embryos for their development and use. These cells are limited in their differentiative capacity, however (10). Induced stem cells are adult cells that have been 'reset' to return to a stem cell state. The 'resetting' is accomplished by the addition of several genes or proteins which returns the cell to to a state resembling an embryonic stem cell in function (11). Clinical trials are underway for both embryonic and adult stem cells.

1.2 Rationale for the Study

Ischemic heart disease is a life-threatening, debilitating condition and is the major cause of death in the world, accounting for nearly 30% of all deaths in 2002 (12). Both gene therapy and stem cells have been used for the treatment of heart disease. *In vivo* gene therapy has been disadvantaged by the increased difficulty of transfecting nondividing cells (8). Genetic modification of cells *in vitro* prior to injection can help to alleviate this

setback, using the modified cells as bioreactors producing the therapeutic protein (13).

Cell therapies have been hampered by the loss of cells at the injury site. Cells require time to attach to a substrate, and many cells can be lost to the surrounding tissues before they are able to join the desired tissue after being injected suspended in saline. An improved delivery method of the cells has the potential to increase the efficacy of the treatment (14). Here the proposed method is the use of a thermoreversible polymers scaffold.

1.3 Hypothesis and Specific Aims

The first hypothesis is that mesenchymal stem cells can effectively be used as a bioreactor for growth factor expression, eliciting an angiogenic response in endothelial cells. The second hypothesis is that a suitable polymer matrix can be found which will allow for the holding of the cells in place, without altering their attributes as stem cells. The final hypothesis is that the combined therapy of modified stem cells in an injectable polymer matrix will show an increased efficacy in treating ischemic heart disease.

Testing these hypotheses was accomplished following these specific aims in conducting the research:

- Plasmid vectors for various growth factors will be constructed and tested for their angiogenic effect on the HUVEC cell line after transfection of MSCs.

- Exploit a polymer matrix to hold the cells in place, and test the cells for any loss of stem cell function, while maintaining gene expression.
- Use the combination of genetically modified stem cells in the polymer matrix in a rat infarct model to evaluate the effectiveness of the treatment.

Chapter 2 in this work gives a comprehensive review of literature covering heart disease, stem cells, cell scaffolds, and gene therapy. Chapter 3 covers the construction of multiple plasmids for use in evaluating the pro-angiogenic effect of various growth factors. Chapter 4 details the work of using PoligoGel as a cell scaffold for use with stem cells. The results for the combined treatment in the rat infarct model are presented in Chapter 5. Chapter 6 describes the future directions in which this research could be taken as well as the significance of the growth factor transfected mesenchymal stem cells for treating ischemic heart disease.

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CHAPTER 2

HEART DISEASE, STEM CELLS, CELL SCAFFOLDS AND GENE THERAPY: LITERATURE REVIEW

This chapter gives an overview of primary research literature pertinent to the work performed and presented here. There are four primary sections dealing with different facets of the research. First, an overview of heart disease, especially ischemic heart disease, is given. This includes the prevalence of the disease with its associated costs, the progression of the disease, and also the current treatment strategies. Next, stem cells as a therapeutic agent are analyzed. Different sources of stem cells are given and compared. Third, the use of cell scaffolds both natural and synthetic are addressed. Finally, gene therapy is examined looking at different techniques for delivering new genes, viral and nonviral, as well common growth factors used for angiogenic treatments.

2.1 Heart Disease

According to the American Heart Association (1) records, in 2006, a person in the United States died every 38 seconds as a result of cardiovascular disease. In fact, 1 in 2.8 deaths in the US is attributed to Cardiovascular Disease (CVD), including heart attacks and stroke. CVD has been the single largest killer in the US every year since 1900 with the exception of 1918. This is not unexpected if one considers that an estimated 1 in 3 Americans suffer from one or more types of CVD. Of the deaths due to CVD in 2004, 52% were due to coronary heart disease, with the remainder being filled in by stroke, heart failure, high blood pressure, arterial disease, and other. If all these major forms of CVD were eliminated, the US life expectancy would rise by close to 7 years; meanwhile, eliminating cancer nets only a 3 year gain. With the continued rise in obesity in the Western world, this trend is unlikely to abate any time soon.

2.1.1 Costs of Heart Disease

Procedures for the treatment of cardiovascular disease numbered almost 7 million in 2005, a 484% increase in 25 years. Catheterization is one of the leading treatments with 1.3 million performed in 2005 at an average cost of just under $27,000 per procedure. 261,000 patients were operated on for bypass surgery, resulting in 469,000 procedures. Heart transplants have been performed at a rate of about 2,200 per year over the past decade, with about 2,700 on the waiting list. Percutaineous Coronary Intervention (PCI, also called

coronary angioplasty) accounted for about 1.3 million procedures in 2005; about 50% on those less than 65 years of age. While there has been a decline in hospitalization for cardiac infarct from the '90s, it is still above levels from the early 1980s (2).

In addition to the loss of life, the monetary cost of treating CVD is estimated to be $502.3 billion for 2010 for both direct and indirect costs. While 38% of those having a coronary attack suffer death as a result in any year, a significant portion of the expenditures is for repeat patients whose treatment has been insufficient to cure the root causes of the disease. In 2008, 770,000 are estimated to have a new coronary attack and 430,000 are expected to have a recurrent attack. Along with the other types of CVD, over 7.2 million inpatient procedures were performed in 2006. Those who have previously survived an acute heart attack are at up to 15 times higher risk for illness and death, leading to additional expenditures treating and retreating the disease.

Risk factors for heart disease are numerous, with the most common being smoking (3), high blood cholesterol, lack of physical activity (4), obesity and excessive weight (http://www.nhlbi.nih.gov/guidelines/obesity/ob_gdlns.pdf), and diabetes mellitus (5). Other factors such as depression have also been shown to potentially contribute to the onset of heart disease (6).

2.1.2 Indications of Disease

By definition, ischemic heart disease is the reduced or lack of blood flow to the heart muscle. Coronary heart disease is the most common cause of this

disease. Often called angina, ischemic heart disease can benefit from early interventions; in the case of a total loss of blood flow, a myocardial infarction occurs, resulting in the loss of viable cells (7). This loss of cells results in a remodeling of the heart. With the infarcted region losing contractile ability, the efficiency of the heart decreases. The volume of the left ventricle increases, and the ejection fraction decreases; this results in a stroke volume that is similar to the uninfarcted heart (8, 9). Larger infarcted regions lead to increased rates of mortality and more significant changes in the heart architecture (10-13). The changes progress over time and can become substantially worse in the years following the ischemic event (9).

Immediately after the triggering of the ischemic event, the now oxygen-deprived cells begin to suffer from nutrient deprivation. In dogs, 38% of the affected cells were nonviable after 40 minutes, and by 24 hours, 85% were non-viable (14). The dead and dying cells can initiate an inflammatory response, which can further damage the remaining tissue as neutrophils are recruited to the area (15). Collagen deposition increases begin at day 2 postinfarct and peak at day 7 (16). By 2 weeks, most of the initial remodeling is finished (17) . The remaining changes are mostly limited to hypertrophy of the remaining cardiomyocytes, and a remodeling of the heart to make the wall stresses more uniform (18). This irreversible damage to the heart occurs in part due to the lack of resident cardiac progenitor cells with the ability to repopulate the damaged site (19).

2.1.3 Current Treatment Options

Treating IHD can be difficult. Generally, current treatment of heart attacks seeks to restrict the size of the ischemic/infracted area, and to treat symptoms. Many additional health issues may be present which can make effective treatment more problematic. One of the most effective treatments can be a lifestyle change, especially quitting smoking, controlling cholesterol levels, and exercise (20, 21). Changing lifestyle has been met with difficulties; some factors creating difficulty for some are a negative attitude toward own age and aging, not being a risk taker, low personal priority on health, health has previously been acceptable, chronic illness, irregular work hours or excessive work hours, and frequent travel (22). Medical treatments for heart disease can be broken down into two classes: pharmaceutical interventions and surgical interventions.

Pharmaceuticals used to treat IHD include alpha-blockers, beta-blockers, calcium channel blockers, nitrates, and others (23). Alpha-blockers are used in the treatment of hypertension (24). Beta-blockers are used in the treatment of arrhythmias (25), and have some use for hypertension (26). Calcium channel blockers in addition to being effective in treating hypertension can be used to control heart rate, and reduce chest pain due to angina pectoris (27). Nitrates are also vasodilators and are also used in the treatment of angina; nitrates have the added advantage of working quickly after administration (28, 29).

Stents and balloon catheters, or bypass surgery, open the blocked vessels to aid in the reperfusion of the effected tissue. This treatment can help

alleviate the symptoms that limit the restriction of blood flow, but are not able to restore heart function by regenerating any lost tissue.

Balloon angioplasty works by the insertion of a balloon catheter into the blocked coronary artery, typically through the femoral artery. Inflation of the balloon compresses the restricting plaque against the arterial walls and stretches the vessel. The stretching can damage the underlying cells, perhaps helping the treated vessels to remain dilated (30, 31).

Stenting the artery works in a similar way. At the time of the forced expansion of the vessel lumen, a metal stent is placed in the artery, holding it open (32). There have been many improvements with the stent, but they are still prone to restenosis, with early stents showing rates of up to 60% (33). All such treatments are also prone to restenosis if repeated to alleviate the symptoms of restenosis (34-37). Even newer drug eluting devices such as the Cypher stent have shown similar trends at longer time points (38).

Bypass surgery involves taking an artery or vein from a different part of the body to graft onto the coronary artery to bypass the blockage in that vessel. While this type of surgery is effective in treating the symptoms of the blockage, it is far more complicated than stenting, or angioplasty (39). Surgery requires the removal of a blood vessel, often from the leg, creating a second wound. The heart is also stopped for the surgery, necessitating cardiopulmonary bypass, which is also not without risk (40).

2.2 Stem Cells

As research into new and more effecting treatments has continued, one area that has seen significant growth in the recent past is the use of stem cells for the treatment of various diseases, including cardiovascular disorders (41-45). Stem cell work has also included bone regeneration (46), cartilage regeneration (47), adipose tissue (48), vascular tissue (49) and more (50-52). Embryonic, mesenchymal, and induced pluripotent stem cells have been used in these types of applications; each brings with it certain advantages and disadvantages. The lack of consensus is due in part to the wide range of culture conditions, and in the case of adult stem cells, the origin of the cells being used (53).

2.2.1 Embryonic Stem Cells

Embryonic stem cells are derived from the inner cell mast of the blastocyst and are capable of generating any of the three germ cell layers. They are self-renewing, and can be cultured indefinitely without changing phenotype (54). When initially isolated, they required the use of a murine feeder layer to maintain an undifferentiated state (55-57). This risks exposure of the cells to animal viruses and is undesirable for future use *in vivo*. More recently this has been overcome, and ESCs can be cultured without the need of a feeder layer (58-62).

The pluripotency of ESCs allows them to become any cell type in the body (63), and potentially replenish any functionality that has been lost in a

tissue or organ. However, also due to this pluripotency, they are also capable of forming teratomas, tumors composed of all three germ layers, limiting their usefulness (54, 64) unless great care is taken to only implant cells that have begun to move down a differentiated pathway (65, 66). While it is possible to select predifferentiated cells for implantation, differentiation along the desired path is typically restricted to only a few percent of the total population, and isolation of the proper cells can be difficult (63, 67).

In addition to the scientific challenges in using ESCs, there are also ethical concerns in using embryonic stem cells (68). Most of the opposition to the use of ESCs is a result of the origin of the cells, viable human embryos. For many, this is viewed as morally wrong and four of the major ethical concerns are: "(1) the moral status of the human embryo; (2) the question of complicity when research relies on the destruction of a developing human life; (3) the moral significance of medically promising alternatives; and (4) the role of law in a pluralistic society." (69) As a consequence of these unresolved moral questions, federal limitations exist for their use in research. These limitations affect the types of research that can be pursued, as well as the availability of funding (70).

ESCs have been studied to replace lost myocardium, and there has been great success in guiding the differentiation to a cardiac-like fate (71, 72). However, *in vivo* problems are seen, such as arrhythmia (73) and teratomas (72). Most therapies involving the use experience similar problems. Geron Corp. is set to begin clinical trials using ESC-derived cells for the treatment of

spinal cord injuries. The trials were delayed for a year to further investigate cyst formation at the site of injection in some of the previous experiments on mice (74). One final concern in using ESCs is the immune rejection of the cells after transplant. While it has been reported that ESCs are not immunogenic (75), this is not as true for differentiated cells, which can cause immune reactions (76, 77).

2.2.2 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (IPS) are another source for cell treatments. IPSs require the addition of several factors to effectively reset them (78). This has been accomplished by the use of viruses (79), but this leaves the possibility of genetic mutation in the cells if lentiviral or retroviral vectors are used. The use of adenoviruses and plasmid construct can be used without the risk of mutation to the genome, but are less efficient (80). More recently, the induction of the pluripotent state has been accomplished without the use of viruses (80-82). IPSs appear to have all the same characteristics that make embryonic stem cells so desirable; omipotency, unlimited self-renewal, etc. They also have the potential of being autologous to the host, removing any concerns over immune response. While promising, this source still requires time for the cells to revert to their stem cell capacity (83), and then to differentiate to the appropriate cell type. Treatment of ischemic disease is best accomplished quickly with irreversible damage occurring within hours, and tissue remodeling in a matter of weeks if there is a total loss of oxygenation,

such as in a cardiac infarction (8, 15, 18, 84). Cells would require collection, induction, differentiation, and then storage until needed at a future event. Fast turnaround can be better accomplished with an adult stem cell.

2.2.3 Adult Stem Cells

Adult stem cells, also called somatic stem cells, or tissue resident stem cells, are multipotent stem cells that reside within fully developed tissues. Their main purpose is to regenerate lost or damaged tissue where they reside (85). Regardless of the tissue source, these cells have been shown capable of differentiating into different cell types, including adipocytes, chondrocytes, and osteoblasts (86-88). Adult stem cells have been isolated from the bone marrow (89), adipose tissue (90), heart tissue (91), dermal layers (92), and also skeletal muscle (93). Most tissues in the body have resident stem cells (94), although harvesting them is not always easy or straightforward. As resident stem cells only account for a small fraction of the total cell population in a tissue, this can require a potentially large biopsy or tissue extraction to secure a large enough population with which to effectively work (85, 95). While many people have sufficient adipose tissue for acquiring enough stem cells for a treatment, it would not be realistic to expect to safely obtain the same level of stem cells from neural, skeletal, or cardiac tissue; the resulting defect left by the biopsy would be problematic. Bone marrow is a source that is relatively simple to obtain and the isolation of the cells does not require mechanical or enzymatic degradation of the sample.

Bone marrow-derived mesenchymal stem cells are typically defined by their lack of CD34, CD45, CD14, and their adherence to solid surfaces(96). There is also a wide variety of other markers and attributes that have been studied and used to mark MSCs (88, 97-101). Complications arise from the fact that there is no definitive marker(s) for stem cells. Some studies have used adherent mononuclear cells from the bone marrow (102); others use side populations isolated by flowcytometery (103) or other methods (98) to further refine the population. As a result, studies use a mixed population of cells that would be nearly impossible to fully characterize.

Differences in the tissues of origin of the different cells can affect the differentiation capacity of the cells, giving preference to one fate more than another (104, 105). Culture conditions, such as substrate hardness, are also able to affect the cell fate and function (106, 107).

In multiple studies where adult stem cells from different sources have been injected into the heart for treating MI, there has been measurable significant improvement in the heart function, and decreases in the infarct size (41, 42, 45). Many of the cells, however, do not have the desired fate *in vivo*. Cell death in these cases is significant, which is to be expected. They are being injected into ischemic regions where cell death has and is occurring as a result of the lack of perfusion to the area. Some have placed the cell loss as high as 90% (108). Cell loss in the studies had been attributed to several causes, including cell death, and cell migration. In addition to the cell loss, the viable remaining cells rarely differentiate into the most desirable cell types,

cardiomyocytes in the case of injection into the heart. Most often, they are seen as endothelial cells, smooth muscle cells, and fibroblasts. This can improve circulation to the surviving tissue; it does not replace the lost function of the defunct muscle tissue.

2.3 Cell Scaffolds

One of the cornerstones of tissue engineering is the use of an appropriate scaffold. Scaffolds must have sufficient mechanical strength for the cells, must be highly porous to allow for cell growth, and should not interfere with tissue function (109). Scaffolds must have sufficient strength to support the cells' growth, but also to mimic the extracellular matrix (110). Porosity of the scaffold is required for nutrient diffusion, as well as integration with host tissues. The ideal scaffold would also be degradable to allow for complete restoration of the native tissues, although with hard tissues, there has also been great success with biomimicry and material integration (111).

In the treatment of MI, research has shown that the injection of a biopolymer gel, such as collagen, improves heart function over nontreated controls. The test animals had thicker ventricular walls, and better ejection fractions. It appears that the addition of a hydrogel to the infracted area limits the remodeling of the tissue that results as part of the wound healing process. This effect was seen without the addition of any other therapeutic measures, such as the inclusion of cells, or growth factors (112).

2.3.1 Natural Hydrogel Scaffolds

Natural scaffolds have included fibrin (112), collagen (113), Matrigel (114), chitosan (115), alginates (116), and cell sheets (117). Natural scaffolds resemble the native extracellular matrix, and are generally well received by the host. While there are 19 or more types of collagen, they all have a similar structure composed of three polypeptide strands. The physical properties can be tuned by adding additional polymers, or by the addition of crosslinking agents (118). The varying of properties has allowed collagen to be used for vascular tissue (119), bone (120), cartilage (121), and others (122, 123). Collagen is a major component of Matrigel as well.

Fibrin has been used for a sealant and adhesive for surgical procedures and is one of the major components in wound healing (124). Fibrin forms by the proteolytic cleavage of fibrinogen by thrombin. Fibrin gels do not exhibit high mechanical strength, limiting the range of possible uses without modification (125). Some of the uses of fibrin gels for tissue engineering include neural (126), cartilage (126), and bone (127) among others.

Chitosan is obtained from the shell of shellfish obtain from renewable resources. In addition to biocompatibility and biodegradability, chitosan has also been shown to have antibacterial (128) and wound-healing activity (129). There are many derivatives of chitosan to alter physical properties and tune it for specific applications (130, 131).

Alginate is obtained from brown algae and has been widely used as a biomaterial both for tissue engineering and drug delivery. It is biocompatible,

has low toxicity, and also has a relative low cost (124). Gelation is achieved by the addition of divalent cations. Unmodified alginate dissolves as a result of the diffusion of the divalent cations from the gel. This can be uncontrolled and unpredictable. As a result, it is often modified to better control the physical properties of the gel (132).

While cell sheets are technically a scaffold-free cellular delivery vehicle, but have great potential for many therapies (133). In addition to single layers of cells, multilayered constructs have been developed with alternating cell types (134). One method of generating cell sheets is growing cells to confluency on thermal-responsive surfaces. By lowering the temperature, the substrate switches from hydrophobic to hydrophilic, and the cells lose the ability to adhere to the surface and can be removed without any enzymatic action on the cells (135). Multilayer constructs have been created and applied to the heart (117, 134) showing great promise.

2.3.2 Synthetic Hydrogel Scaffolds

Synthetic hydrogel scaffolds can be tuned for whatever requirements are needed for a particular application: crosslinking density (136), pore size (137), hydrophobicity (137), gelation mechanism (138, 139), degradation rates (140), and other definable properties (141). While many of these properties can also be modified with natural polymers, synthetics have no predefined characteristics, such as the backbone of chitosan, or alginate which may be modified but remain intact. Many of these modifications are accomplished by

combining natural and synthetic materials, blending the distinction between the two classes. Some of the most common synthetic materials are poly(acrylic acid) (PAA), poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), Polyphosphazene, and synthetic polypeptides. Poly(acrylic acid) has a structure that can be easily modified by the inclusion of different side chains on the monomers. Two of the most common PAA-based polymers for tissue engineering are poly(2-hydroxyethyl methacrylate) (HEMA) and poly(N-isopropylacrylamide) (PNIPAAm) (124). HEMA has been used for the microencapsulation of cells (142) as well as cartilage reconstruction (143). PNIPAAm has been used for spinal cord repair (144), cell encapsulation (145), and responsive surfaces for cell sheet engineering. PNIPAAm is a thermoresponsive polymer. The homopolymer undergoes a phase transition at 32 degrees Celsius, moving from aqueous to a gel. Modification of the polymer by the inclusion of comonomers: hydrophilic monomers such as acrylic acid can raise the LCST (146), hydrophobic monomers like butyric acid can lower the LCST. The use of pH sensitive groups can also lead to additional environmental sensitivities to the polymer (147).

Poly(ethylene glycol) has been widely used as a biomaterial not just as a hydrogel or scaffold, but also to modify small molecules to increase their stability, circulation time, making them less prone to proteolytic inactivation, and lowering toxicity, among other benefits (148). PEG is hydrophilic, non-toxic, chemically stable, and has modifiable hydroxyl end groups. In use as a scaffold, methods need to be used to crosslink the chains together. Acrylate

end groups are one common method of accomplishing this, allowing for photo initiation of the crosslinking reaction (149). One of the reasons PEG has been so widely used is the relative low cost and wide commercial availability of different molecular weights, different end groups, and different branching structures; Sigma-Aldrich currently carries over 300 different PEG-based products. One of the major uses of PEG is combining it with different materials to form a composite material. Examples of copolymers include poly(propylene oxide) to create the poloxomers (150), poly(lactic-co-glycolic acid) (151), polyesters (152), as well as many natural polymers (153-155). PEG-based and PEG-containing hydrogels have been used in many applications, including the brain (156), orthopedically (157), and with cardioprogeneter cells (158).

Hydrogels of poly(vinyl alcohol) are typically crosslinked with gluteraldehyde (159), or epichlorohydrin (160). These monomers can be toxic and must be washed from the gel prior to implantation (124).

Polyphosphazenes have the advantage of being able to alter the degradation kinetics through modification of side groups, and not the backbone (161). Polyphosphazenes have also been modified (162-164) and used in many applications, such as skeletal regeneration (165) and cell encapsulation (166).

2.4 Gene Therapy

The American Society of Gene and Cell Therapy defines gene therapy as: “The introduction or alteration of genetic material within a cell or organism with the intention of curing or treating a disease.” Diseases are typically

treated by either replacing defective genes (167), by providing new genetic instructions (168), or modifying the current expression of genes (169) for the recipient cells. As of 2007, over 1340 clinical trials had been competed, approved, or started involving gene therapy (170). The first began in 1989 (171) treating melanoma with genetically modified lymphocytes transfected with a retrovirus. Monogenic diseases such as hemophilia (172), cystic fibrosis (173), and severe combined immunodeficiency (SCID) (174, 175) are all caused by a genetic mutation resulting in a single nonfunctioning protein. The administration of the proper gene can cure the disease by providing the required native protein. Cancer (176), ischemia (176, 177), cardiovascular disease (178), chronic wounds (179), and more have also been treated using different growth factors and proteins to elicit the appropriate response from the host.

There are two broad categories of agents for the delivery and transfection of the target cells: viral, and nonviral. Viral gene therapy replaces the pathogenic genes of a virus with therapeutic genes. In most cases, the virus is rendered replication deficient (180), but there are instances were *in vivo* viral replication is desirable (181). Viral vectors can trigger an immune response in the host, which has been fatal (182). Even if there are no immediate consequences, some may appear later, such as leukemia as a result of genomic integration (183), or immune response on subsequent administrations (184). Despite the drawbacks of using viral agents for gene therapy, they are extremely efficient, especially when compared to nonviral

methods (185). Nonviral gene therapy makes use of cationic polymers, non-ionic polymers, lipids, mechanical interactions, and any other method that does not require a virus. Nonviral gene therapy generally does not have immunological complications, allowing for repeat administration, also, preparation of the particles is simpler, especially on a large scale, and is easily modifiable.

2.4.1 Viral Gene Delivery

Viruses can be very diverse; they may contain either DNA or RNA either single or double stranded, the genetic material may integrate or not, and they may be enveloped or not (183, 184, 186). In the case of replication-competent viruses, they may be lytic, bursting the cell on release, or lysogenic. The most common viruses used in clinical trials for gene therapy are retroviruses (including lentivirus), adenoviruses, poxvirus, adeno-associated virus, and the herpes simplex virus (183).

Retroviruses were the first viral vectors used for gene therapy. Retroviruses contain an RNA genome up to 8kb in size. The viral genome is transcribed into DNA and incorporates into the host genome. This allows for persistent expression of the delivered gene; however, depending on the location of the insertion, this can be oncogenic. Most retrovirus vectors only transfect dividing cells; the lentivirus is able to transfect nondividing cells (183, 187).

Adenoviruses have been modified to reduce their immunogenic potential (188, 189). After entering the cell, the viral particle is carried to the nucleus where the double stranded DNA is transcribed. The DNA does not integrate with the host, but its genome is limited to less than 36 kb (187). The biggest drawback to adenoviruses is their high immunogenicity.

Adeno-associated viruses are dependent on adenoviruses for replication. They have a persistent transfection, but can integrate into the host genome (190). These agents can only carry small amounts of DNA, less than 5kb.

Herpes simplex virus is very potent at transfecting neural cells, where the transfection is persistent. Like adenoviruses, they are highly inflammatory, but they can carry up to 150 kb (183).

2.4.2 Nonviral Gene Therapy

Due to the immunogenic and mutation risks associated with viral gene therapy, nonviral alternatives have been researched. While viruses have evolved to overcome barriers to transfection and transcription of their genome, synthetic approaches still must overcome multiple barriers to transfect cells. First, they must protect the DNA as it is moving to the cell. The polyplexes must be stable and protected from nuclease degradation. Second, they must travel to the appropriate tissue and enter the desired cell. Third, once in the cell, they must be able to escape the endosome. Finally, after release from the endosome, the DNA must make it to the nucleus before it can be transcribed.

2.4.2.1 Overcoming Barriers to Transfection

DNA/polymer polyplexes need to find a balance between protection of the DNA, and being able to release the DNA in the cytoplasm. If the complex is too loose, it may uncomplex in the extra cellular space, or in the case of systemic injection, in the blood. This exposes the DNA to nucleases and degradation (191). However, if the complexes are too tight, the DNA will not be released in the cell (192).

Entrance to the cell is block by the lipid bilayer that comprises the cell membrane. The surface of the cell membrane carries a negative charge, making association of positively charged particles favorable through electrostatic interactions (193). Particles are then internalized by phagocytosis (194). In addition to electrostatic interactions, targeting is also effectively used for membrane association. Antibodies or ligands can be attached to the polyplex, allowing the particles to be internalized by receptor- mediated endocytosis (195). Lipoplexes are also potentially able to deliver their genetic material through membrane fusion. Membrane fusion inducers, such as spermine and chloroquine, can improve cellular entry (196, 197).

The most commonly proposed method for cationic polymers to escape the endosome is the proton sponge effect. This hypothesis is that as the endosome pH drops by the addition of protons, the protons are absorbed by the nitrogen on the polymer, leaving the counter ion concentration to swell and increase the osmotic pressure in the endosome. This leads to a rupture of the endosome, releasing the polyplex into the cytoplasm (198). Fusion peptides

and membrane fusion inducers can also be used to aid in escape from the endosome (199).

Nuclear entry is perhaps the most challenging barrier, especially in non-dividing cells. In a dividing cell, the nuclear membrane dissolves during mitosis, allowing for the vector to be in the nucleus when the nuclear membrane reforms(200). In nondividing cells, nuclear localization signals can be used to aid in transport to the nucleus (201, 202).

2.4.2.2 Cationic Polymers for Nonviral Gene Therapy

Polyethylenimine (PEI) is the standard for other polymers to compare against. Both linear and branched PEI effectively condense DNA into uniform particles with sizes around 100nm (203). The structure of PEI is such that every third molecule on the backbone is nitrogen; this yields a highly charged polymer that strongly interacts with the phosphate groups on DNA (204). The high charge has been associated with toxicity of the complex (205). There are many derivatives of PEI that have tried to lower the toxicity of native PEI. Some of these modifications include PEGylation (206), cholesterol (207), and succinylation (208).

One derivative of PEI that has been used is WSLP, which stands for water-soluble lipopolymer. Here a branched PEI with a molecular weight of 1800 was conjugated with cholesterol chloroformate. The resulting construct is both effective and safe for use with a variety of cell types. It has been shown to be highly efficient, but does not exhibit the toxicity associated with higher

molecular weight PEI (207). This polymer has been used many times *in vivo* to treat a range of diseases, including cancer (209), ischemic myocardium (210), erectile dysfunction (211), and neural disorders (212). This polymer has been effective in the delivery of both plasmid DNA, as well as siRNA (213).

Poly(l-lysine) (PLL) is another cationic polymer, and was one of the first to be used for gene therapy (214). It is a linear polymer of the amino acid lysine, which makes it biodegradable; however, high molecular weight samples show high toxicity. It is, however, quickly bound by serum protein *in vivo* and rapidly cleared. PLL is also a poor transfection agent without modifications, such as the inclusion of chloroquine or other fusogenic agents (215). PLL has been modified with PEG to improve circulation times (216), and also by substitution of histidine residues in the backbone to enhance the proton sponge effect after cellular uptake (217).

A more recent polymer family constructed for use in gene therapy contains a bioreducible backbone with a disulfide bond. This class of polymer is polycationic and therefore able to complex DNA and protect it from degradation outside of the cell. However, after entering the cell and escaping the endosome, the cell reductive potential inside the cell breaks the disulfide bonds, allowing for the release of the DNA (218). Many different polymers with the reducible backbone have been shown to be effective. CBA/DAH, which is composed of cystaminebisacrylamide (CBA) and 1,6-diaminohexane (DAH), shows high efficiency and low toxicity and has been used for the delivery of both DNA and siRNA (219, 220). ABP is an arginine-grafted bioreducible

polymer based on CBA/DAH. ABP showed improved transfection with reduced toxicity. This improvement may be due to localization to the nucleus, and not related to cell penetration (221). Other polymers with CBA in their backbones include TETA (222), pegylated TETA (223), and other small libraries of polymers (224).

In addition to these well-studied polymers, others have focused their efforts on constructing large libraries of polymers that have yielded some potentially useful polymers for gene therapy (225).

2.4.2.3 Other Methods of Nonviral Gene Therapy

Cationic liposomes are another class of delivery vehicles. Common cationic lipids for inclusion in the liposome include DOTMA, DC-CHOL, and DOSPA; these are then mixed with DOPE or another neutral phospholipid. DNA/lipid complexes are also usually not as well condensed, and are also prone to aggregation; as such, they are often combined with cationic polymers (226, 227).

Other less-often-used delivery methods include naked DNA(228), non-ionic polymers (229, 230), and mechanical methods, including electroporation, ultrasound, and pressure perfusion (231).

2.4.3 Growth Factor Gene Delivery

Delivering genes coding for growth factors is a growing area of research. VEGF gene delivery alone has been used to treat a variety of illnesses,

including poor bronchial circulation (232), erectile dysfunction (233), hind limb ischemia (234), transplanted fat tissue (235), as well as others.

2.4.3.1 VEGF

The VEGF family of proteins is highly conserved in a wide range of animals, from fish to mammals (about 68% of the amino acids are identical); homologs can be found in even less evolved creatures such as fly, nematode, and jellyfish (236). VEGF-A is the most common and contains eight exons; alternative splicing yields at least six different expressed isoforms with a 165 amino acid being the most prevalent and active. All of the VEGF proteins are excreted. VEGF acts on two different receptors, VEGFR-1, and VEGFR-2; the signaling pathways for these two receptors are significantly different. Both receptors are tyrosine kinases, but the exact signaling pathway has not been conclusively determined. Both are required receptors for development; mice lacking VEGFR-1 or VEGFR-2 do not develop in utero past day 9.5. There is agreement that VEGFR-2 is the more prominent receptor for angiogenesis and proliferation. Treatments involving VEGF have already gone to clinical trials (237), and have also been delivered to the myocardium both as a plasmid (238), as well as via transfected myoblasts (239).

2.4.3.2 FGF

There are two major forms of FGF, FGF-1 and 2, also referred to as acidic and basic, respectively. FGF influences both proliferation and migration,

but uses different signaling pathways (240). FGF is also required during embryonic development of the heart. Despite FGFs inducing a strong angiogenic response, it is generally accepted that they require VEGF to promote angiogenesis (241). FGF has been effectively used in treating ischemic disease despite potentially requiring additional factors for best results (242).

2.4.3.3 PDGF

PDGF is a dimeric protein with four expressed proteins, and five isoforms: -AA, -BB, -AB, -CC, and –DD. The different isoforms react preferentially with one of two receptors: PDGF receptor alpha and beta. While isoforms of PDGF composed of –A and –B are excreted from the cell in active form, -C and –D require proteolytic cleavage in the extracellular space to be activated. PDGF-C has the following effects: “stimulates fibroblast proliferation, epithelial migration, extensive vascularization and neutrophil infiltration” (243). The two receptors can also dimerize, giving three different receptors. Each of the five PDGF isoforms has a different activation pattern on the receptors (244). PDGF has also been shown to effectively treat heart disease (245).

2.4.3.4 IGF

IGF exists in two forms, IGF-I and IGF-II, each with differences in the signaling output after binding with their receptors. IGF-I has been shown to stave off apoptosis acting at several points (246). IGF-II can promote

differentiation, proliferation, and is also upregulated in myocytes during differentiation (247). There has long been a known association between IGF and angiogenesis (248).

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CHAPTER 3

PLASMID DNA CONSTRUCTS FOR FGF, IGF, AND PDGF-CC AND MSC TRANSFECTION

3.1 Abstract

The first step in gene therapy is selecting the gene to be used and preparing the construct to be used for transfection. Plasmid DNA was utilized for its therapeutic effect. Presented here is the work related to the construction of plasmid DNA encoding three different growth factors: fibroblast growth factor (FGF), insulin-like growth factor (IGF), and platelet-derived growth factor - C (PDGF-C). These genes were inserted into the pCI plasmid backbone, which contains the CMV (cytomegalovirus) promoter, SV40 (Simian vacuolating virus 40) poly(A) tail, and an ampicilin resistance gene. An alternate, hypoxia-induced promoter, RTP801, was also used with the PDGF-C gene. Multiple polymers were also tested for use in transfecting mesenchymal stem cells, including the use of nuclear localization signal conjugated polymers. After multiple studies, it was found that branched poly(ethyleneimine) (PEI) with a molecular weight of 25 kDa and an N:P ratio of

10:1 was the most efficient polymer for transfection. Transfection persisted past 1 week while declining in intensity after 48 hours. MSCs transfected with growth factors were able to induce growth responses in both HUVEC (human umbilical vein endothelial cell) and A7R5 (rat smooth muscle cell) cell lines, indicating the effectiveness of the plasmid constructs. An *in vitro* angiogenesis assay, and proliferation assays, were utilized to help in the determination of which growth factor should be used in downstream applications. Based on the results presented here, PDGF-C was chosen for further use.

3.2 Introduction

Plasmid DNA (pDNA) for the transfection of cells has been researched for decades (1). It has low toxicity, does not integrate into the host genome, is free of any potential viral contamination, and is relatively easy to produce on a large scale (2). While pDNA alone, often referred to as naked DNA, has been used with some success, it is more often combined with polymers or lipids for enhanced gene delivery (3-5).

Plasmids are typically small, circular, double stranded DNA molecules that are separate from chromosomal DNA, and are capable of replication. Plasmids are produced by introducing the desired plasmid into *Escherichia coli* bacteria, fermentation of the bacteria, and then harvesting the pDNA from the cells (6). Many companies produce kits to allow for the purification of pDNA in quantities ranging from minipreps for isolating up to 20 µg, to gigapreps, which can be used for the isolation of up to 10 mg of product.

All plasmids for cloning share some of the same features. They require an origin of replication to allow for autonomous replication, and a gene for antibiotic resistance to allow for the selection of cells containing the plasmid. The third feature is the expression cassette, which includes the desired gene, a promoter, and a polyadenylation sequence, also referred to as the poly(A) tail region, which is useful for stability, nuclear export, and translation (7). While not a required feature, most plasmid vectors also have a multiple cloning region, a sequence with multiple sites where 10 or more restriction enzymes are active (8).

Restriction enzymes cleave DNA at very specific sites, often leaving "sticky ends," unpaired nucleotides at the cut site. Restriction enzymes have had an incredible impact in molecular biology and medicine. The Nobel Prize for Physiology or Medicine was awarded to Daniel Nathans, Werner Arber, and Hamilton Smith in 1978 for their work with restriction enzymes (9). A DNA fragment can be inserted into a plasmid by cutting both DNA molecules with the same restriction enzymes and allowing for the combination of the two at the cut sites. The cut DNA is then repaired by the use of another enzyme, ligase. Using this method, it is possible to express any DNA sequence in the plasmid backbone (10).

In addition to restriction enzymes, the development of polymerase chain reaction (PCR) has been instrumental in allowing for the cloning of therapeutic genes into plasmids. The basis of PCR is in the Taq Polymerase, an enzyme for DNA polymerization isolated from thermophilic bacteria capable of surviving

temperatures above 90 Celsius, temperatures required for the denaturation of double stranded DNA to single strands. There are three steps for PCR. First is denaturation of the DNA to single strands. Second is annealing; the primers can then attach to the parent strands of DNA by hydrogen bonding. The third step is elongation, or the creation of the complimentary strand of DNA. These steps are cycled, resulting in exponential growth of the target sequence (11). Kary Mullis was awarded the 1993 Nobel Prize in Chemistry for working out the principles of PCR a decade earlier (9).

When the desired gene is known, there are several ways that the plasmid can be optimized for production of the gene; the promoter can be modified or changed, the poly(A) tail can be modified, or elements of the backbone of the plasmid can also be modified. Promoters can be cell type-specific (12), hypoxia-responsive (13), insulin-responsive (14), or responsive to any other physiological or environmental cue (15, 16).

The delivery of the plasmid DNA must also be considered. Condensation of the plasmid by polymers ensures protection from nucleases and efficient cellular uptake if designed well. After entry into the cell, the plasmid must enter the nucleus in order for translation to occur, ultimately leading in production of the desired gene. The use of a nuclear localization signal (NLS) was attempted here to improve transfection efficiency. Other groups have reported some success in the use of NLS peptides conjugated directly to the DNA (17), as well as with liposomes (18). The results vary greatly based on many factors including conjugation levels, cell type, and gene carrier.

The growth factors being investigated vary in potency and in their effects on different cell types. One method of measuring their efficacy is an *in vitro* angiogenesis assay (19). The use of recombinant protein allows for the comparison in a controlled environment, although care must be taken, as the conditions can be very different *in vivo*. One of the more common assays is performed by sandwiching cells in a biological hydrogel scaffold, often Matrigel or fibrin, and then quantifying the response of the cells (20).

The effects of growth factors on stem cells have been studied and yielded some conflicting results. It is generally agreed that growth factors will encourage proliferation, survival, and migration of stem cells, although with varying degrees of efficiency for the different factors. The propensity of the growth factors to induce differentiation is less understood. In the comparison of growth factors with similar signaling cascades, PDGF and epidermal growth factor (EGF) show a 90% overlap in proteins phosphorylated in human MSCs after exposure to the growth factors, yet only EGF elicited an osteogenic response (21). This was found to be the result of a single protein, phosphatidylinositol 3-kinase (PI3K). Meanwhile, the growth factor HGF was indicated to encourage differentiation to a cardiomyocyte phenotype (22). However, the differentiation was not complete, and in the authors own words "further differentiative steps probably require additional factors." Other studies showed that FGF would induce osteogenic differentiation, but in cells treated with dexamethasone (23).

It is unlikely that cells will differentiate into any cell type with only the addition of a single growth factor; at least it has not been documented. In the present study, the use of growth factors is not intended as a differentiative agent to push the stem cells to any of the potentially beneficial cell types, but is only intended to promote cell proliferation and survival in both the native cells as well as the implanted cells. As the MSCs integrate into the host tissue, they will have retained the potential to become endothelial cells, smooth muscle cells, cardiac cells, or other cell types, all based on the signals received at the injection site.

3.3 Materials and Methods

3.3.1 Materials

Restriction enzymes and the empty pCI plasmid backbone were obtained from Promega (Madison, WI). Terrific broth was purchased from Fisher Scientific (Pittsburgh, PA). T4 ligase, PCR reagents including Taq Polymerase High Fidelity, Pfx polymerase, and SuperScript® III RTS First-Strand cDNA Synthesis Kit were obtained from Invitrogen (Carlsbad, CA). Agarose gel for electrophoreses was purchased from Promega (Madison WI). Purification of PCR samples and extraction of DNA fragments from agarose gel was accomplished using the Wizard® SV Gel and PCR Clean-Up System from Promega (Madison, WI). A cDNA library extracted from human placenta was purchased from Invitrogen(Carlsbad, CA). RNA was isolated using the PureLink™ RNA Mini Kit from Invitrogen (Carlsbad, CA). Plasmid purification was performed by

either the PureLink™ HiPure Plasmid Miniprep Kit or the Maxiprep Kit purchased from Invitrogen (Carlsbad, CA). The GENEMATE Quanti-Marker 1kb DNA ladder was used when running an agarose gel purchased from BioExpress (Kaysville, UT). DNA concentrations were measured on a NanoDrop UV-Vis from Thermo Scientific (Wilmington, DE). DH5-alpha competent cells and S.O.C. media were purchased from Invitrogen (Carlsbad, CA). Polyethylenimine was obtained from Sigma (St. Louis, MO). All cell culture media and reagents were obtained from Invitrogen. A plasmid encoding VEGF in the pCI plasmid backbone had previously been constructed and was used as supplied.

The NLS peptide sequence used is n-PKKKRKVEDPYC-c, which is based off the SV40 virus, and has a molecular weight of 1490.79 Daltons. The crosslinker used was sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) obtained from Pierce Protein Research Products (Rockford, IL). WSLP (Water-Soluble Lipopolymer) was synthesized in house as previously described (24). Deuterated water for NMR and PEI were purchased from Sigma (St. Louis, MO). WSLP, a PEI derivative was synthesized previously by our group, as published(24).

Fibrin and thrombin were purchased from Sigma (St. Louis, MO) and were cell culture grade. Recombinant human PDGF-CC was purchased from R&D Systems (Minneapolis, MN). $VEGF_{165}$, PDGF-BB, bFGF, HGF, and IGF were all purchased from PeproTech Inc (Rocky Hill, NJ).

3.3.2 CMV-PDGF-CC Plasmid Construction

The CMV-PDGF-CC plasmid was constructed by placing the gene sequence for PDGF-CC in the pCI backbone plasmid at the HindIII and XbaI restriction sites. The PDGF-CC gene is 1038 base pairs long and was isolated from cDNA generated from human placental tissue, which is rich with myriad growth factors. The cDNA template was amplified using primers specific for PDGF-CC; the 5' primer was ATA AGC TTC TCA GCC AAA TGA GCC CTC and the 3' primer was GCT CTA GAC TAT CCT CCT GTG CTC C. Primers were designed to include restriction sites to the ends of the gene for insertion into the plasmid.

Amplification was carried out according to the manufacturer's instructions. In brief, reagents were mixed to the following final concentrations in distilled water:

PCR buffer – 1x, dNTPs - 0.2mN, MgSO4 – 2mM, Primers – 0.2 µM each, template DNA 1 µL, Platinum Taq High Fidelity – 1 unit.

The following cycles were followed: initial denaturation – 2 minutes, 30 cycles of denaturation - 30 seconds, annealing - 30 seconds, extension - 1:15 minutes, and a final extension of 5 minutes followed by holding at 4 degrees.

The results of the PCR amplification were purified by running on a 1% agarose gel with a 1kb Quanti-Marker ladder for reference. The band around 1000kb was removed from the gel and purified using the Promega Gel cleanup kit. The purified fragment and empty pCI plasmid were then digested separately using HindIII and XbaI enzymes in a double digestion. The digestion

was allowed to proceed for 4 hours using the recommended conditions, including the use of acylated BSA. Following the digestion, the samples were purified using Promega's Wizard® SV Gel and PCR Clean-Up System. Concentrations were then measured using the NanoDrop, allowing for a 3:1 insert to plasmid molar ratio. The DNA was then allowed to react overnight at 4 degrees with the T4 ligase for completion of the plasmid. DH5 alpha competent cells were transformed using S.O.C. Medium according to the recommended protocol. Cells were then placed on a LB agar plate treated with ampicilin (100 micro grams/mL) for screening of positive colonies.

Five colonies were grown in 5 ml of TB broth for plasmid isolation and further analysis. Colonies in TB broth were allowed to grow overnight and pDNA was then collected using the Miniprep Kit. Plasmids were then run on an agarose gel to verify the correct plasmid size. Results can be seen in Figure 3.1; the two positive clones were verified by restriction enzyme digest and sequencing.

3.3.3 RTP801-PDGF-CC plasmid construction

After successful cloning of the CMV-PDGF-CC plasmid, the gene was inserted into a plasmid with a RTP801 promoter that shows higher response in hypoxic conditions. The gene was removed from the CMV-PDGF-CC construct at the HindIII and XbaI restriction sites and isolated by gel electrophoresis. The band for the gene was removed from the gel and purified with a gel cleanup kit. The empty RTP801 plasmid was digested with the same restriction

enzymes and purified with a PCR cleanup kit. The two fragments were then combined in a 3/1 molar ratio and ligated according to the supplied protocol. DHa-5 cells were transformed and selected on ampicilin agar plates. Identification of proper insertion of the gene into the plasmid backbone was confirmed by gel electrophoresis by looking at the size of the finished construct, as shown in Figure 3.2. Empty plasmids and double insertions show incorrect sizes. The two plasmids with the correct molecular weight were further confirmed by digestion.

3.3.4 FGF Plasmid Construction

The CMV-FGF plasmid was constructed by placing the gene sequence for FGF in the pCI backbone plasmid at the HindIII and XmaI restriction sites. The FGF gene is 476 base pairs long and was isolated from cDNA generated from CADMEC cells that had been stimulated with VEGF. RNA from the CADMEC cells was isolated using the PureLink™ RNA Mini Kit from invitrogen. The cDNA template was amplified using primers specific for FGF; the 5' primer was TTA AGC TTC TGG TGG GTG TCG GGA, and the 3' primer was GTC TAG ATC AGC TCT TAG CAG ACA TTG G. Primers were designed to introduce restriction sites to the ends of the gene for insertion into the plasmid.

Amplification was carried out according to the manufacturer's instructions as previous constructs. The following cycles were followed: initial denaturation – 2 minutes, - 30 cycles of denaturation - 30 seconds, annealing -

30 seconds, extension - 45 seconds, and a final extension of 5 minutes followed by holding at 4 degrees.

The results of the PCR amplification were purified by running on a 1% agarose gel with a 1kb Quanti-Marker ladder for reference. The band around 500bp was removed from the gel and purified using the Promega Gel cleanup kit. The purified fragment and empty pCI plasmid were then digested separately using HindIII and XbaI enzymes in a double digestion. The digestion was allowed to proceed for 4 hours using the recommended conditions, including the use of acylated BSA. Following the digestion, the samples were purified using Promega's Wizard® SV Gel and PCR Clean-Up System. Concentrations were then measured using the NanoDrop allowing for a 3:1 insert to plasmid molar ratio. The DNA was then allowed to react overnight at 4 degrees with the T4 ligase for completion of the plasmid. DH5 alpha competent cells were transformed using S.O.C. Medium according to the recommended protocol. Cells were then placed on a LB agar plate treated with ampicilin (100 µg/mL) for screening of positive colonies. Positive colonies were grown in 10 mL of TB broth from which the plasmid was purified for analysis. Verification of positive clones were verified by restriction enzyme digest and sequencing. The plasmid map is shown in Figure 3.3.

Sequencing of the plasmid showed two mutations in the FGF sequence, one of which altered the amino acid sequence of the protein and was corrected by site directed mutagenesis. Primers for site directed mutagenesis were used as provided from University of Utah core facilities and were complimentary to

each other. The 5' primer was CCG TTA CCT GGC TAT G(A)A GGA AGA TGG AAG ATT AC with the base in parenthesis being changed from G to A. The 3' primer was the reverse compliment of the 5' primer. Pfx polymerase was used for its higher fidelity. Multiple reactions were carried out using different quantities of template DNA to ensure optimal conditions. PCR was run with an initial 2 denaturation at 94 degrees, followed by 12 cycles of 15 seconds for denaturation at 95 degrees, 1 minute at 55 degrees for annealing, and 6 minutes at 68 degrees for elongation. PCR samples were then purified and subjected to digestion with a Dpn I restriction enzyme. Dpn I only recognizes and cuts methylated DNA produced in the bacteria; the synthetic DNA produced during PCR is not cut, leaving only the altered DNA. The resulting plasmids were used to transform XL-1 blue cells. Two colonies were obtained, one of which had the desired mutation back to the correct sequence.

3.3.5 Construction of CMV-IGF Plasmid

The CMV-IGF plasmid was constructed by placing the gene sequence for IGF in the pCI backbone plasmid at the HindIII and XbaI restriction sites. The IGF gene is 462 base pairs long and was isolated from cDNA generated from human placental tissue. The cDNA template was amplified using primers specific for IGF; the 5' primer was AGA AGC TTG CAA TGG GAA AAA TCA G, and the 3' primer was ATC TAG AGG GTC TTC CTA CAT CCT G. Primers were designed to introduce restriction sites to the ends of the gene for insertion into the plasmid.

Amplification was carried out according to the manufacturers' instructions as with previous plasmids. The following cycles were followed: initial denaturation – 2 minutes, - 30 cycles of denaturation - 30 seconds, annealing - 30 seconds, extension - 45 seconds, and a final extension of 5 minutes followed by holding at 4 degrees.

The results of the PCR amplification were purified by running on a 1% agarose gel with a 1kb Quanti-Marker ladder for reference. The band around 500 bp was removed from the gel and purified using the Promega Gel cleanup kit. The purified fragment and empty pCI plasmid were then digested separately using HindIII and XbaI enzymes in a double digestion. The digestion was allowed to proceed for 4 hours using the recommended conditions, including the use of acylated BSA. Following the digestion, the samples were purified using Promega's Wizard® SV Gel and PCR Clean-Up System. Concentrations were then measured using the NanoDrop, allowing for a 3:1 insert to plasmid molar ratio. The DNA was then allowed to react overnight at 4 degrees with the T4 ligase for completion of the plasmid. DH5 alpha competent cells were transformed using S.O.C. Medium according to the recommended protocol. Cells were then placed on a LB agar plate treated with ampicilin (100 micro grams/mL) for screening of positive colonies. Positive colonies were grown in 10 mL of TB broth from which the plasmid was purified for analysis. Verification of positive clones were verified by restriction enzyme digest and sequencing. A map of the plasmid construct can be seen in Figure 3.4.

3.3.6 NLS to Polymer Conjugation

The conjugation of the SV40 NLS peptide to WSLP took place in the following manner. 5mg of bPEI was dissolved in deionized water and the pH was adjusted to 7.4 by the addition of dilute HCl. The crosslinker sulfo-SMCC (1.7 mg) was dissolved in 0.25 mL DMF and added to the polymer. SMCC was allowed to react with WSLP for 90 minutes at room temperature where the NHS ester on the crosslinker reacts with primary amines on the polymer. After the crosslinker and polymer had reacted, the NLS peptide (10.8 mg) was added to the reaction vessel and allowed to react overnight. The resulting polymer was then purified by dialyses for 3 days against deionized water with frequent replacement of the dialysate.

3.3.7 Cell Culture

MSCs were cultured in MEM-alpha supplemented with 20% FBS and 1x antibiotics. When the cells reached 80-90% confluency, they would be split 3-1. Media is exchanged every 3-4 days. Passaging cells was accomplished by the removal of the growth media, washing twice with PBS. The addition of Tripsin 0.25% and EDTA was added and allowed to incubate at 37 degrees for 5 minutes, or until the cells had lifted off the plate. Cells were used prior to passage 10.

3.3.8 Transfection

For transfection, cells are plated on standard tissue culture surfaces and allowed to grow to 50% confluency or less. Standard growth media was replaced with serum-free media and the DNA complexes were added. After 4 hours, the media was exchanged with standard growth media. Cells were allowed to incubate as normal until further analyzed.

DNA complexes were formed at a final DNA concentration of 0.05 mg/mL. Two µg of DNA were added to one well on a standard six well plate, with 2 mL of media in the well: roughly one microgram per 100,000 cells. Complexes were formed by mixing a polymer solution with a DNA-containing solution. DNA solutions were composed of 5% glucose in water and DNA at a concentration of 0.10 mg/mL. Polymer solutions were 5% glucose in water with a polymer concentration appropriate for creating the proper N/P ratio for the complex. Typical N/P ratios are from 5:1 and up, depending on the polymer being used. A 10:1 N/P ratio for PEI would require a polymer concentration of 0.03 mg/mL.

3.3.9 Cell Viability

Cell viability was measured by an MTT (MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assay. Cells were grown and transfected as described in Section 2.2.7. After incubation for 24-48 hours, MTT was added to a final concentration of 0.2 mg/mL. Cells were allowed to incubate for 3-4 hours to allow the MTT to be converted to an insoluble formazan crystal. After

incubation, the media was aspirated off the cells and DMSO was added to dissolve the crystals. The concentration was then measured on a spectrophotometer reading at 570nm.

3.3.10 *In Vitro* Angiogenesis Assay

Fibrin gels were formed by first reconstituting fibrinogen and thrombin at concentrations of 10 mg/ml and 100 units/ml, respectively. These solutions were then mixed in a variety of final combinations from 6.6 mg/ml fibrinogen to 1.5 mg/ml, and final thrombin concentrations from 40 units to 1 unit, to see which formed the most transparent gel for ease of analyzing the cell structures. The optimized fibrin gel contained 4 mg/ml fibrin, and 40 units of thrombin, and was used for the assay. HUVECs were placed in a 96 well plate above 50 ml of the fibrin gel and allowed to grow overnight. A second layer of 50 ml was then placed above the cells. Once the gel was set, supplemented growth media was added to the wells. Media was supplemented with high and low concentrations of the growth factor of interest. Cells were analyzed following 72 hours of growth. Analysis was performed by taking a photograph of the cells in a random field in each well and looking at the morphological structure of the cells; each field was scored from 0-5. The scores breakdown as follows: 0 = no cell interactions, 1 = minimal cell migration and alignment, 2 = capillary tubes begin to form, 3 = sprouting of new tubes visible, 4 = closed polygons of cells visible, and 5 = a complex mesh of closed structures visible.

The results are the average of 6 scored images and were analyzed by a one-way ANOVA with Bonferroni posttests.

3.4 Results

3.4.1 Plasmid Construction

All of the plasmids were successfully constructed as verified by sequencing of the gene of interest, as well as by restriction enzyme digestion. Genes showed perfect alignment to sequences given in the NIH GenBank database for IGF and FGF; the accession numbers are as follows: for IGF NM_000618, for FGF NM_002006. The accession number for PDGF-C is NM_016205.2 and initially showed a single base mutation altering the amino acid sequence. Site-directed mutagenesis performed on the CMV-FGF plasmid was successful; sequencing the gene a second time showed perfect alignment with the published sequence.

3.4.2 NLS Conjugation

The conjugation of the NLS peptide to the WSLP backbone yielded 3.5 mg of final product, a 20% yield based on the total weight of all the reactants. NMR was used to calculate the conjugation ratios of polymer to NLS peptide. WSLP has peaks around 2.5 to 3.0 corresponding to the hydrogen on the PEI backbone, while the tyrosine residues on the NLS peptide show peaks at 7.1 and 6.7. Comparing the ratios of these peaks, the estimated conjugation ratio is 59%. The estimated molecular weight based on the conjugation ratio is 2874

g/mol. The NMR spectrum for the purified product can be seen in Figure 3.5. All peaks resulting from the tyrosine on the peptide were assumed to be chemically conjugated, as free peptide would have been lost during the dialysis purification of the polymer.

3.4.3 Polymer Transfection

Multiple polymers were tested for ability to transfect and also for cellular toxicity. Some of the polymers used were branched and linear PEI, WSLP, and NLS conjugated PEI with N:P ratios ranging from 5:1 to 30:1 depending on the polymer. Results for the MTT assay and luciferase transfection can be seen in Figure 3.6. Error bars on the graph are the standard deviation with n=3. The experiments were conducted on 6-well plates.

As expected, increasing polymer concentration resulted in higher toxicity within polymer groups. Gene expression also increased with polymer concentration with the NLS and PEI polymer groups; WSLP did not show increased transfection, most likely due to the significant toxicity on these cells. Highest transfection with a luciferase reporter gene plasmid was seen with PEI at an N:P ratio of 20:1 ($p<0.001$). This combination also showed significant toxicity. The best choices were PEI at 10:1 or WSLP at 10:1. Repeat experiments using GFP expressing plasmid, and flow cytometry analysis, showed PEI 10:1 outperforming WSLP 10:1 both in luminescence and in the percentage of cells transfected, and both being upstaged by the NLS

conjugated PEI at 20:1, as seen in Figure 3.7. The NLS-conjugated polymer had significant toxicity based on the flow cytometry data; as a result, PEI 10:1 was used for future experiments.

Transfections were also shown to persist with gene expression decreasing, but continuing, past 7 days, as shown in Figure 3.8. Days 1 and 2 were significantly different from day 7 ($p<0.05$). Changing the oxygen levels also gave the anticipated result with gene mRNA levels dropping with the CMV promoter under hypoxic conditions and rising with the use of the RTP801 promoter, data shown in Figure 3.9. Here the hypoxia-responsive promoter only gave a two-fold increase in expression levels, and the changes were not statistically significant.

3.4.4 *In Vitro* Angiogenesis Assay

The *in vitro* angiogenesis assay was accomplished by sandwiching HUVECs between two layers of a fibrin gel. Gels with final concentrations of 4 mg/ml fibrin and 40 units of thrombin were observed to have the best optical properties for visualizing the cells in the gel. In the initial assay, all of the growth factors were used at two different concentrations, 1.5 times the ED50, and 3 times the ED50. VEGF and PDGF-C were both found to produce more efficient responses, and were further used with transfected MSCs. The results for the HUVECs exposed to transfected MSCs are given in Figure 3.10, showing PDGF-CC and VEGF each outperforming the other growth factors with no statistical difference between the two.

3.4.4 Response to Expressed Genes After Transfection

In order to test the ability of the expressed proteins to generate a growth response, transfected stem cells were placed in well inserts over human umbilical vein endothelial cells (HUVECs); results are given in Figure 3.11. The growth factors FGF, VEGF, and PDGF-C, as well as a Luciferase plasmid, were all tested and compared to cells with just media. PDGF-C was statistically different from all other groups with a p-value of < 0.001 as calculated with ANOVA and Bonferroni posttest. As mature blood vessels contain more than just endothelial cells, tests were repeated using A7R5 cells, rat aorta smooth muscle cells; results are given in Figure 3.12. However, for this test, media was transferred from one well to another, as opposed to using inserts.

3.5 Discussion

The transfection of cells has two primary concerns. First are the characteristics of the gene expression, predominantly the duration of expression and the expression level. If expression drops off from the elevated levels too quickly, there may not be enough time for an appropriate response to take place. If the elevated gene expression is too high, or persists too long, the unwanted side effects can occur, such as the formation of angiomas when working with growth factors. It is also possible for the local concentration of the growth factor to become high enough to saturate cell receptors, interfering with a cell's ability to sense the direction of the gradient, and as a result may

not be capable of responding to the signal. The second factor is toxicity caused by the polymers used for delivery. Cationic polymers have been shown to be toxic, and are more toxic with increased molecular weight and concentration. However, larger polymer chains and higher concentrations are more efficient at condensing DNA. The balance must be found between expression levels and toxicity. Here that balance was found to be with PEI at an N:P ratio of 10:1. Different cell lines will show different sensitivities to some polymers, and must be looked at individually.

Testing of various polymers with the MSCs indicated that bPEI was the most effective polymer for transfection. While the NLS containing polymer showed higher transfection efficiency, with an excreted protein, total production is of more importance than the number of producing cells. The toxicity associated with the higher concentrations was also a deterrent to using the NLS polymer. While all polymers tested were able to transfect the MSCs to some degree, none outperformed branched PEI.

The hypothesis that the NLS peptide would improve transfection was correct in that a larger population of cells did express the gene. But taken as a whole, the cell population was not producing the protein at higher levels, and for a protein released from the cell, total expression is of greater relevance than the number of producing cells. While the direct cause was not studied, it was noted that the use of the NLS polymer resulted in only minimal numbers of outliers producing multiple times higher signals for GFP signals when compared to PEI. Refinements to the NLS-polymer conjugates were attempted, but

without added success. Different conjugating agents were tried, specifically sulfo-SMCC and PEG 3400. The peptide was conjugated to both PEI and WSLP. Despite these different attempts, there was no significant improvement over the base polymer for any of the combinations. While these results were disappointing, most others have also been met with limited if any success (25).

Plasmids for the expression of multiple growth factors were created and tested in MSCs and on HUVEC cell lines. The creation of plasmids driven by the CMV promoter and encoding the growth factors PDGF-C, FGF, and IGF was satisfactorily completed based on the sequencing of the genes. The ability of the plasmids to elicit a positive growth and angiogenic response as a result of transfection also confirmed the positive construction of the plasmids. While both PDGF and VEGF showed angiogenic responses, both as indicated by the angiogenesis assay as well as HUVEC proliferation, only PDGF showed a positive response for smooth muscle cells. In the A7R5 smooth muscle cells, VEGF-treated cells actually showed lower proliferation than the control cells. With the exception of capillaries, mature blood vessels require both endothelial cells as well as smooth muscle cells. For the revascularization of the heart, which is the end goal, blood vessels larger than capillaries will be required. PDGF was chosen for further study as an outcome of the results presented here. In the case that PDGF proves inadequate at a later point in the study, VEGF would be the replacement candidate of choice. As referenced earlier, VEGF has been used successfully in generating an angiogenic response, both in the heart and elsewhere in the body.

While less desirable, it would also be a possibility to inject both cells and plasmid DNA complexes at the same time into the infarcted region. This would potentially provide the benefit of both the stem cells, as well as an increased cell population for transfection resulting in protein production. Also of note is that when transfecting resident cells, they are unlikely to wash from the area, meaning that protein produced by them will be concentrated at the site of the injury.

3.6 Conclusion

PDGF was designated as the best growth factor with which to proceed for further study. While this is based on the results of multiple experiments, it is also exciting due to the novelty of working with PDGF-C which has not been known and studied as long as other growth factors, such as VEGF or FGF. The plasmid will be delivered after complexation with branched PEI 25k. While disappointing that the NLS conjugations were in vain, PEI has a long and proven track record in conjugations, and while toxicity is a concern with this polymer, cell death was minimal and the concentration of 10:1. Additionally, the transfection will be done *in vitro* prior to injection of the cells, so systemic toxicity will not be an issue.

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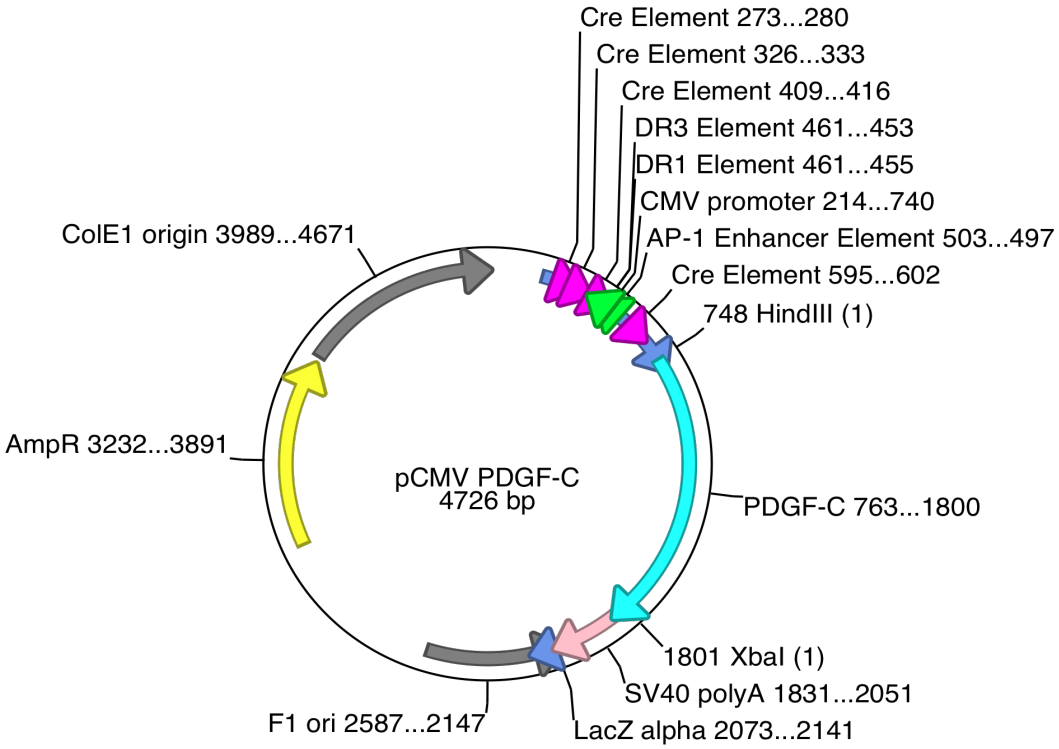


Figure 3.1 Schematic presentation of the pCMV-PDGF-C plasmid showing the ampicilin resistance (AmpR), the F1 and ColE1 origins of replication, the CMV promoter and cAMP response elements, the IGF gene, restriction enzyme cut sites, and the poly(A) tail from the SV40 virus.

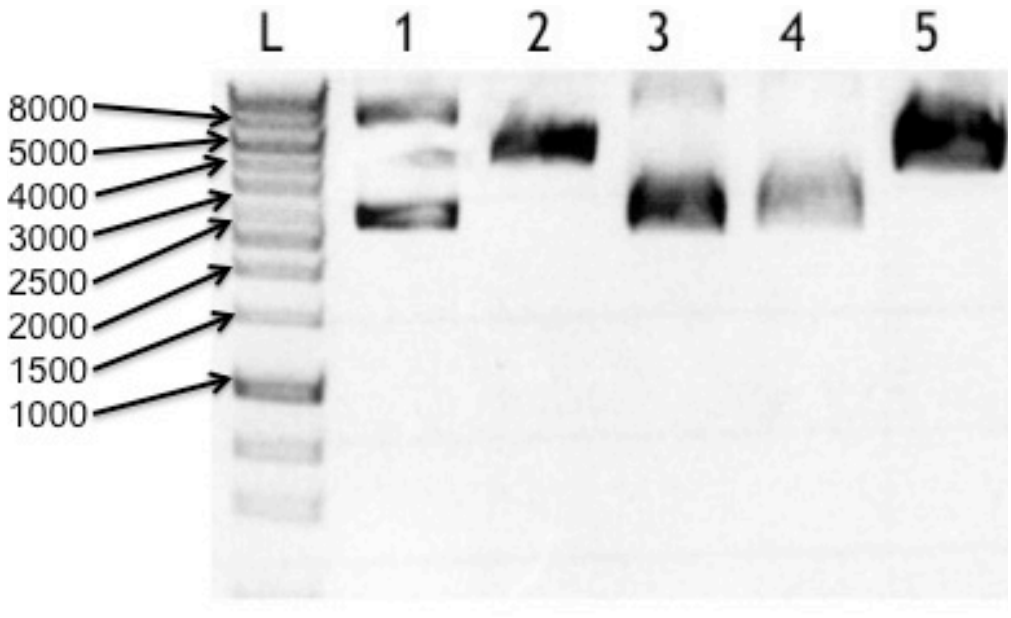


Figure 3.2 Gel electrophoresis of plasmids obtained from positive colonies. L = DNA Ladder (number indicates base pairs), 1-5 indicates different positive selected colonies. 2 and 5 demonstrate a single band at the anticipated size (~4700). Lane 1, 3, and 4 show bands corresponding to an empty plasmid.

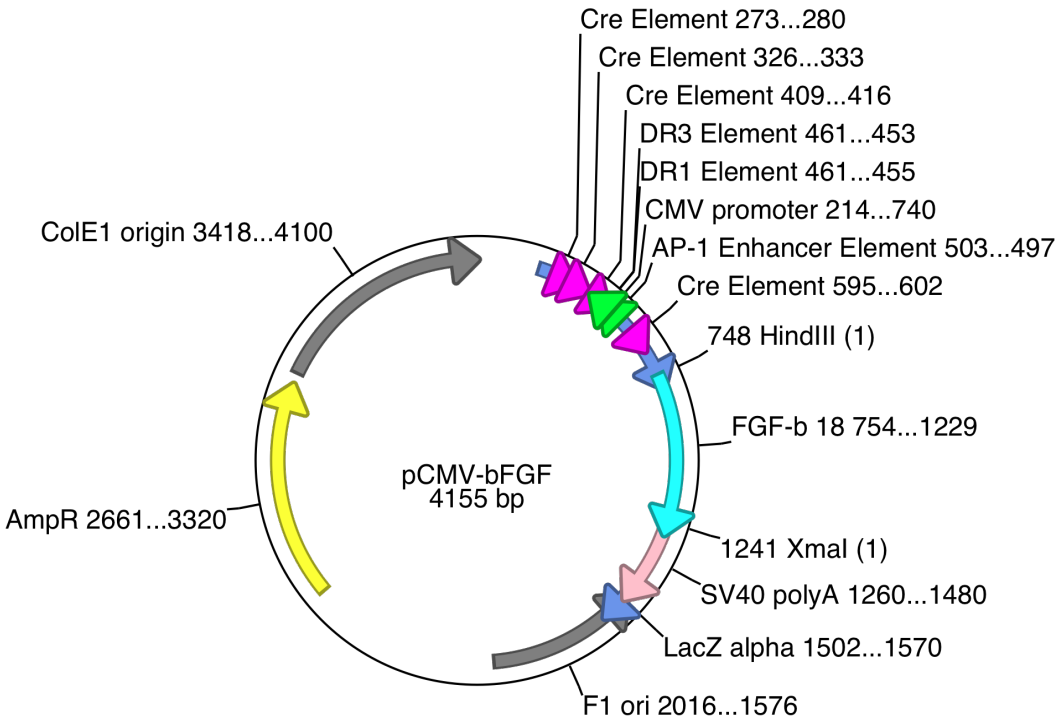


Figure 3.3. Schematic presentation of the pCMV-FGF plasmid showing the ampicilin resistance (AmpR), the F1 and ColE1 origins of replication, the CMV promoter and cAMP response elements, the FGF gene, restriction enzyme cut sites, and the poly(A) tail from the SV40 virus.

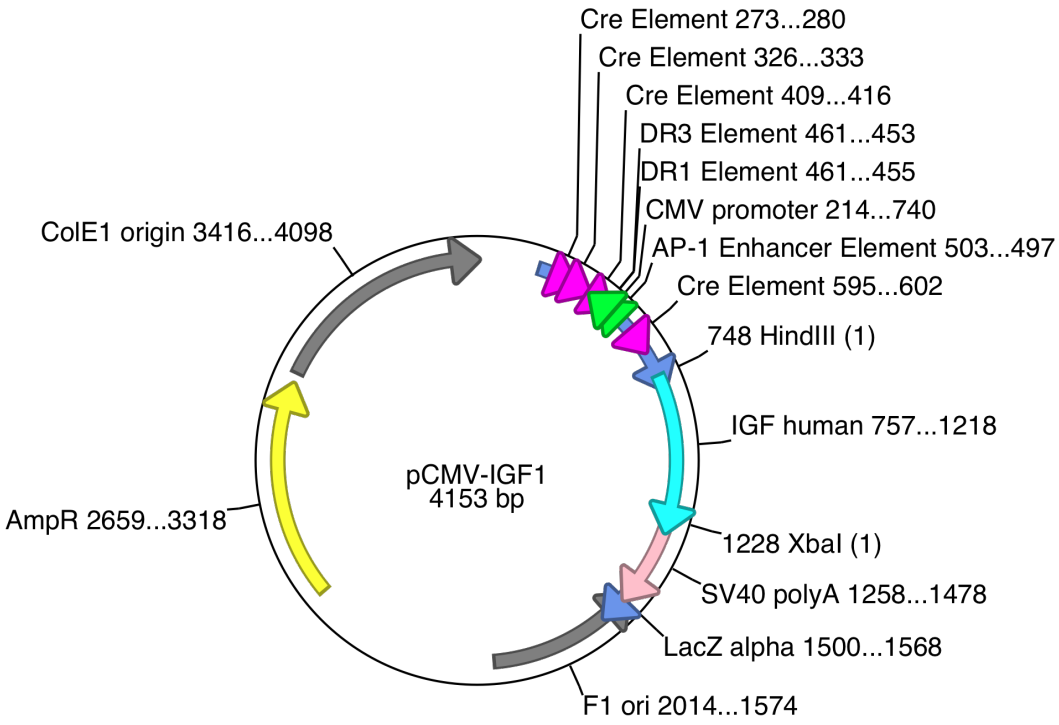


Figure 3.4 Schematic presentation of the pCMV-IGF plasmid showing the ampicilin resistance (AmpR), the F1 and ColE1 origins of replication, the CMV promoter and cAMP response elements, the IGF gene, restriction enzyme cut sites, and the poly(A) tail from the SV40 virus.

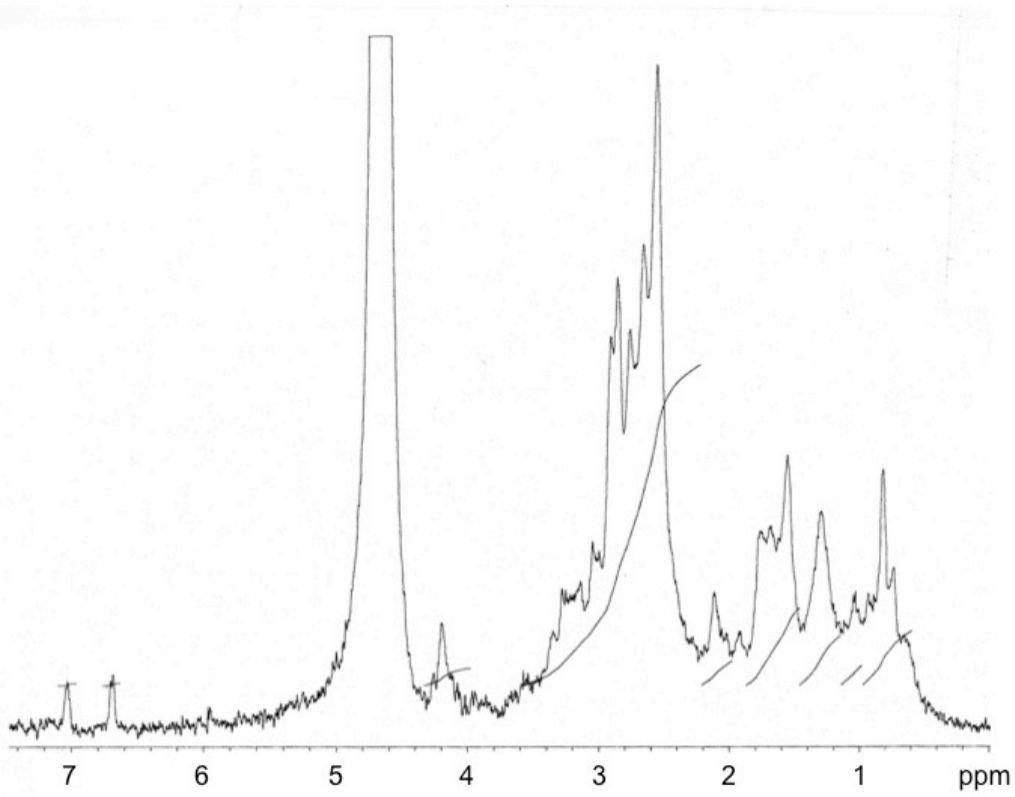


Figure 3.5 NMR spectrum of NLS conjugated to WSLP. The peaks at 7.1 and 6.7 correspond to the tyrosine residues on the NLS peptide, the peaks from 3.5 to 2 are from the PEI, and the peaks between 2 and 1 are from the cholesterol residue on the WSLP.

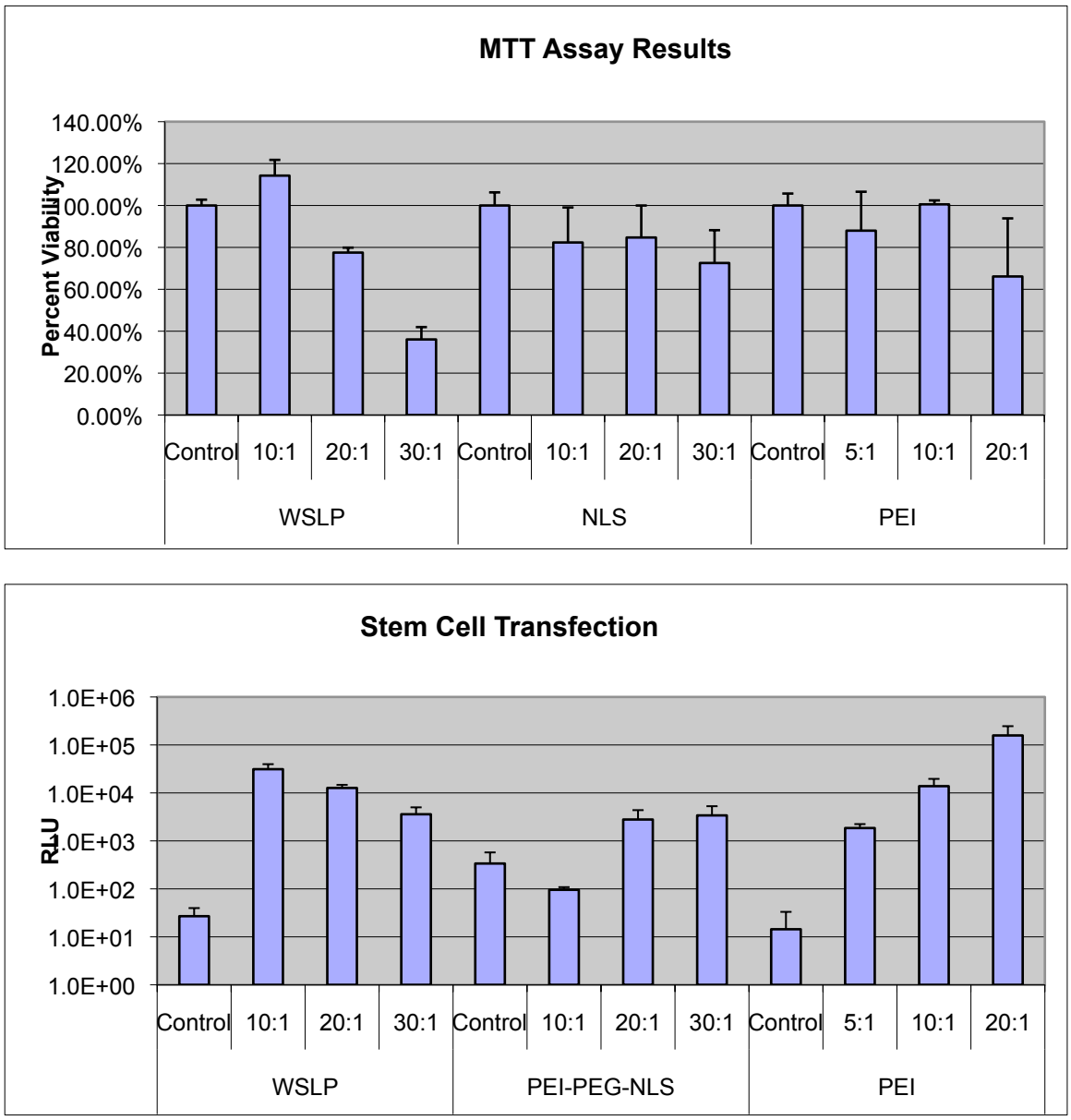
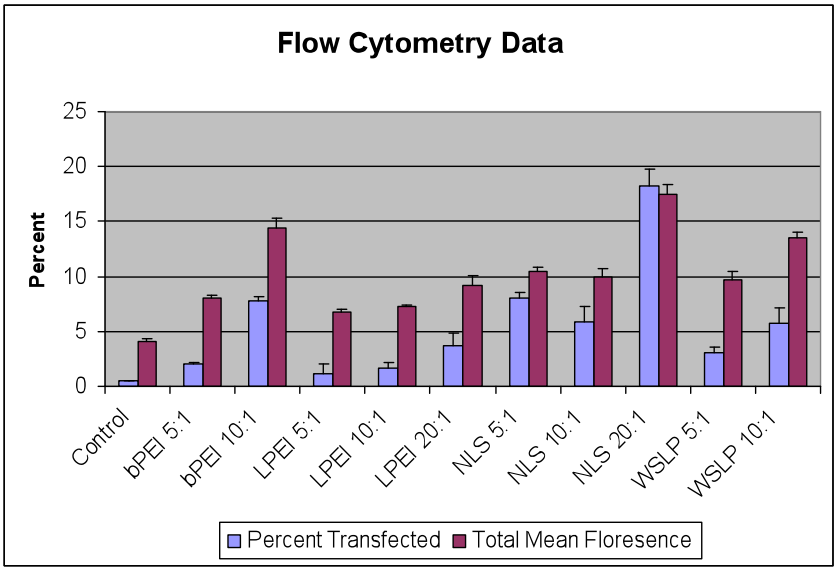


Figure 3.6 Toxicity (top) and luminescence (bottom) after transfection of MSCs with a CMV-luciferase plasmid complexed with multiple polymers and multiple N:P ratios. Viability measured by MTT assay and normalized to control wells. Luminescence is reported as Relative Light Units (RLU) where measured light intensity has been normalized to total protein content. Error bars are the standard deviation; n=4.

A.



B.

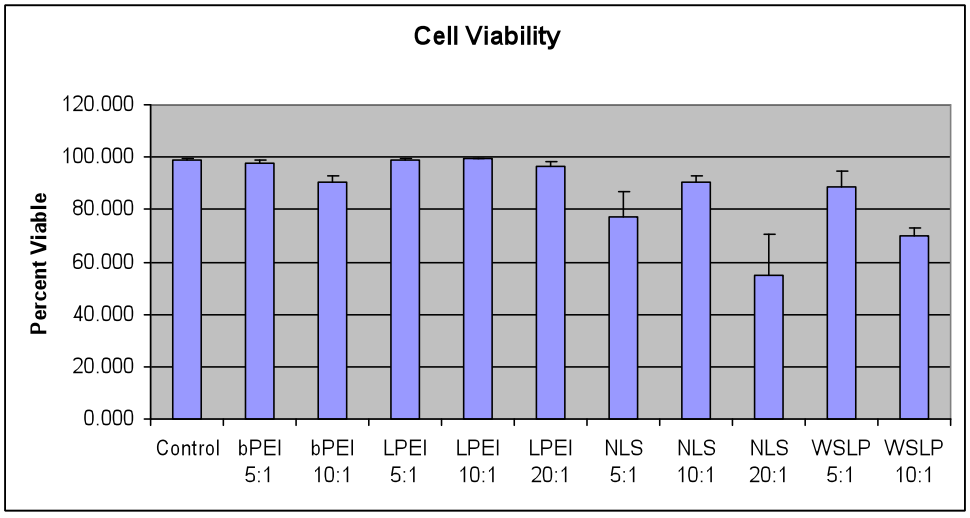


Figure 3.7 Comparison of polymer conjugates for transfection and toxicity. A) MSCs transfected with a CMV-GFP plasmid showed highest transfection with NLS conjugated PEI 25k 20:1 based both on the total percent of the cells transfected as well as the total florescence. There was no statistical difference between bPEI and the NLS conjugation. B) Despite the higher transfection, the NLS PEI also had higher associated toxicity for the 20:1 conjugation ($p<0.05$). Error bars are the standard deviation with n=3.

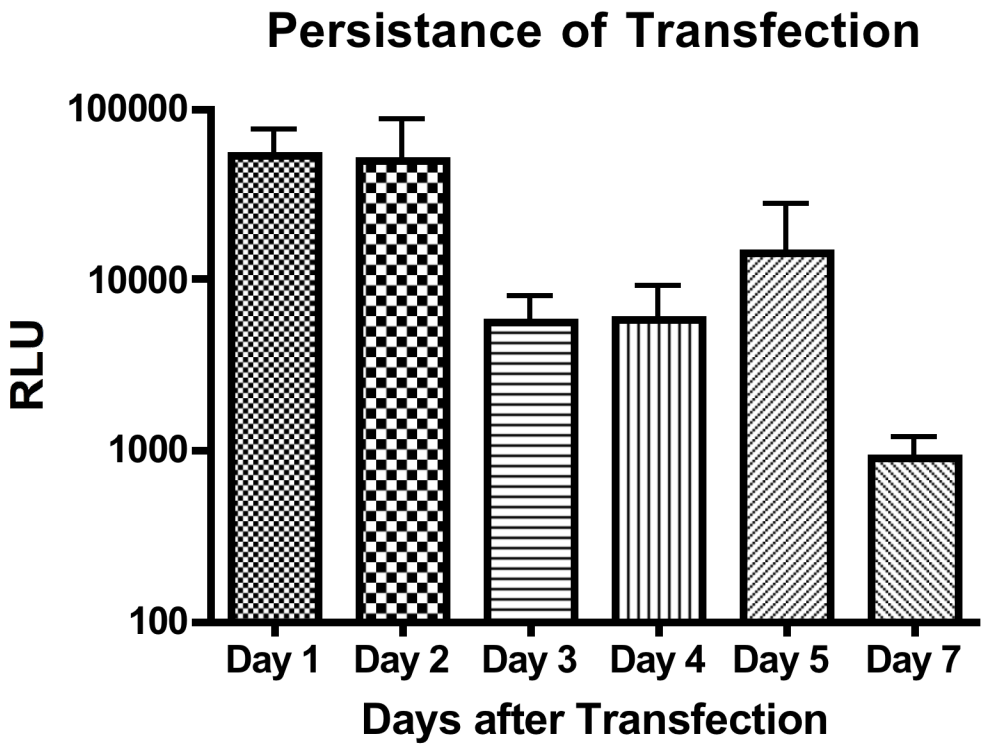


Figure 3.8 Transfection of MSCs persisted past 7 days when transfected with a CMV-luciferase plasmid. Relative Light Units (RLU) is the total luminescence divided by the total protein concentration (mg). Error bars are the standard deviation with n=4.

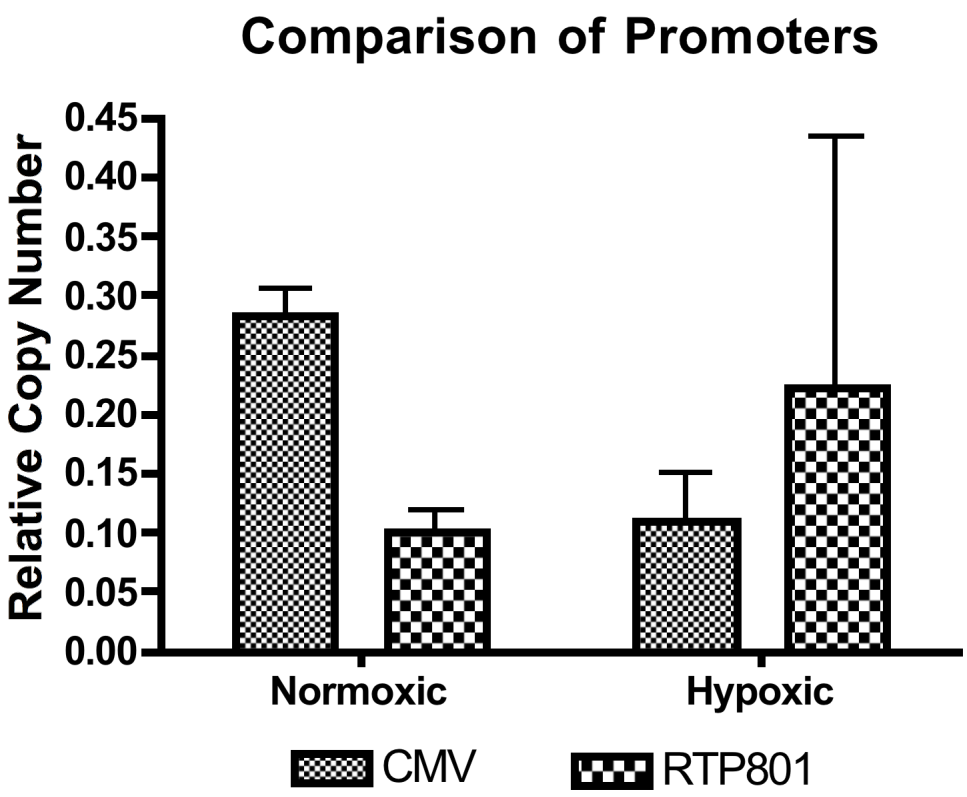


Figure 3.9 The RTP801 promoter is hypoxia responsive and results in upregulated gene expression when the cell experiences hypoxic conditions. Here it is shown to roughly double the expression of PDGF in MSCs. Data were collected by PCR comparing to beta-actin. Error bars are the standard deviation with n=3.

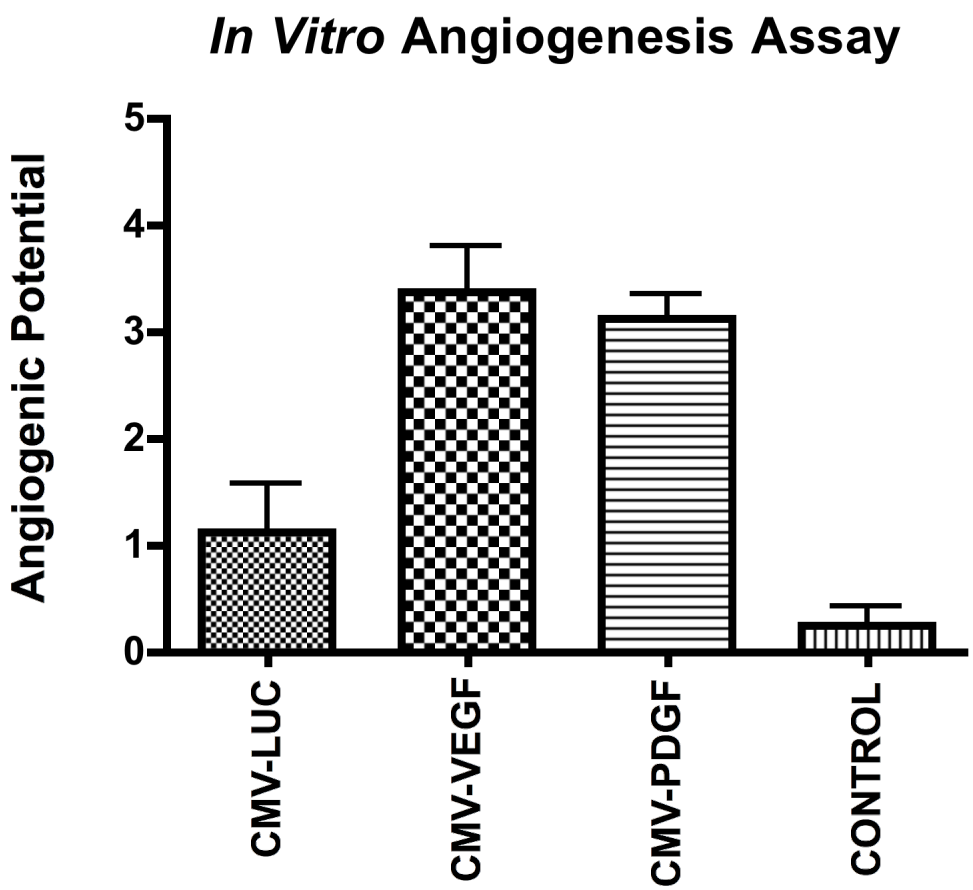


Figure 3.10 Angiogenic potential of different growth factor as recombinant protein. HUVECs were sandwiched between layers of fibrin. MSCs were transfected with growth factor encoding plasmids, embedded in PoligoGel and placed over the HUVECs. Cells were allowed to grow for 72 hours; no media was replenished during that time. After the incubation period, MSCs were washed off with cold PBS and the HUVECs were imaged and cell organization was analyzed and quantified. The scores breakdown as follows: 0 = no cell interactions, 1 = minimal cell migration and alignment, 2 = capillary tubes begin to form, 3 = sprouting of new tubes visible, 4 = closed polygons of cells visible, and 5 = a complex mesh of closed structures visible.

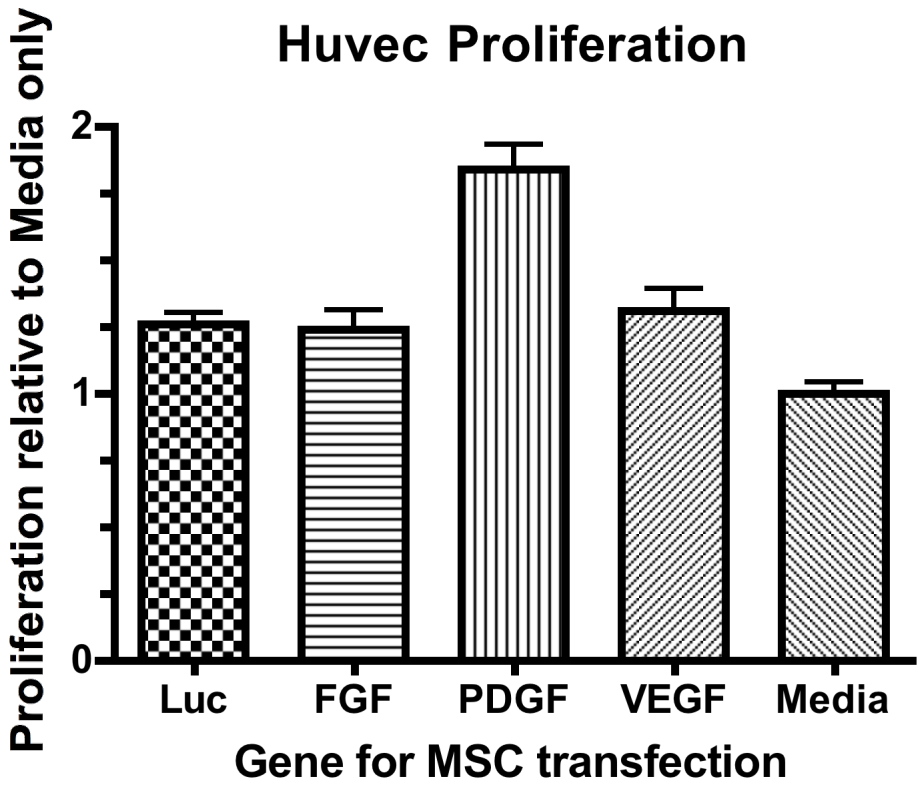


Figure 3.11 HUVECs co-cultured with transfected MSCs were shown to grow most significantly in the presence of PDGF-CC transfected cells. PDGF-C cells were significantly different from all others with $p<0.001$, as measured by ANOVA with Bonferroni posttest. Error bars are the standard deviation with n=4.

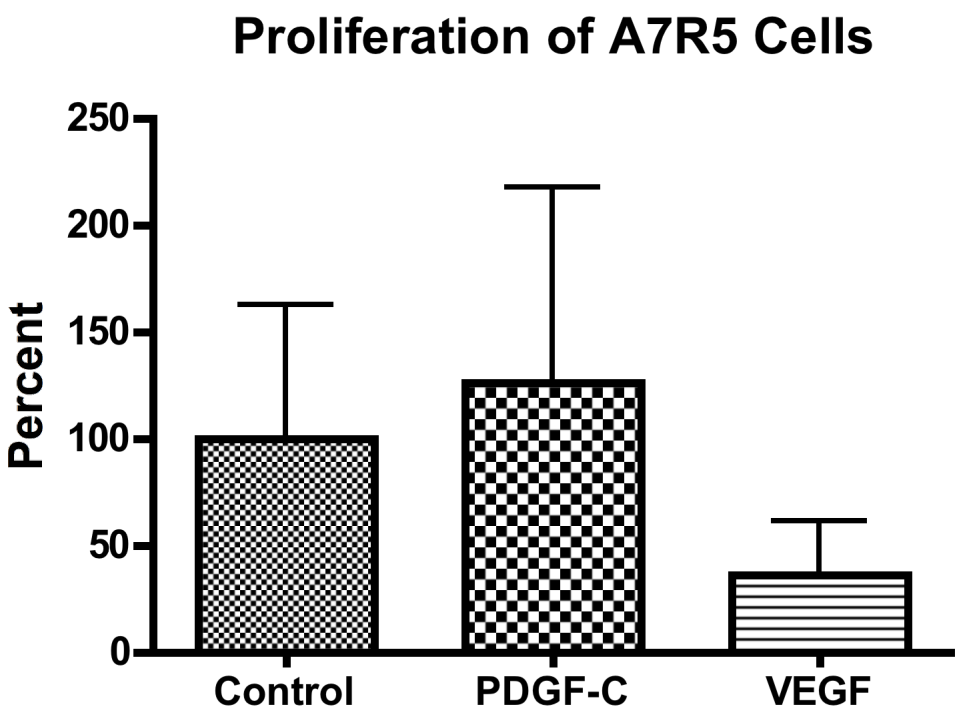


Figure 3.12 Proliferation of A7R5 rat aorta smooth muscle cells with media transfer from transfected MSCs. Media was transferred twice over 3 days. The control group was defined as 100% growth, and the growth factors were compared to that group. Error bars are the standard deviation with n=3. There was no statistical difference as measured by ANOVA.

CHAPTER 4

POLIGOGEL AS A CELL SCAFFOLD

4.1 Abstract

The concept of stem cells as a therapeutic agent has been gaining momentum. A common mode of administration of these cells is by direct injection into the target tissue. This can result in many of the cells being lost due to reflux from the injection site, leading to a local loss of implanted cells. PoligoGel is a nontoxic hydrogel with an LCST near body temperature. It is also shown to be nontoxic to multiple cell types, and in the case of rat mesenchymal stem cells does not alter their differentiative capacity, either by inducing differentiation, or limiting the potential for subsequent differentiation after removal from the gel. Embedding cells in PoligoGel also does not interfere with the cells ability to delivery therapeutic growth factors post-transfection with plasmid DNA. Here a thermoresponsive hydrogel, PoligoGel, is shown to have potential to act as a scaffold for the retention of cells at an injection site, mitigating migration or washing of the cells away from the target site after implantation.

4.2 Introduction

Cell-based treatment for disease is growing in scope and effectiveness. Uses currently range from neural therapies (1), to heart treatments (2), and to cartilage (3). This has increased the need to develop delivery strategies that are able to maximize the potential of these cellular treatments. This is especially true in the case of autografts, where limited numbers of cells can be isolated and a fast turnaround time is often key (4). While the use of cells suspended in saline is commonly demonstrated in studies, it is not perfect; only a limited number of cells may engraft at the implant site. One study found only 10% of injected cells were retained 30 minutes after a myocardial injection (5). The authors concluded by commenting on the need of furthering the development of strategies to retain the cells where needed.

Bone marrow-derived MSCs have been known for over a decade to have the potential for differentiating into multiple cell types (6). This cell type can be obtained from the bone marrow by aspiration, resulting in a large number of collected cells that do not require harsh enzymatic treatment to isolate from the host tissue, or intensive isolation procedures. In many instances, all that is required is simply taking the adherent fraction of mononuclear cells; this does result in some heterogeneity in the population, however (7).

Scaffolding systems seeded with cells is one method for limiting the departure of implanted cells from the target area. Seeded scaffolds have been accomplished by the use of preformed scaffolds (8), or injectable *in situ*

forming scaffolds which may be stiff (9) or more often a hydrogel (10, 11). Cell sheets have also been used with success (12).

Hydrogels are ideal candidates for soft tissue as they are supple, and generally perform well from a biocompatibility perspective. Hydrogels can also be tailored by altering the crosslink density, the pore size, degradability, stiffness, and other parameters in order to achieve the requisite properties (13). Hydrogels have long been used as a biomaterial for local deliver of small molecules, including examples from the late 1970s (14). Stimuli-sensitive polymers have also been used in this fashion (15). More recently, *in situ* gelling hydrogels have been used to deliver various molecules such as steroids, proteins, and chemotherapy agents (10, 16-18). Materials undergoing gelation *in situ* are especially useful due to the ease of delivery, a simple injection. Hydrogels which gel via chemical crosslinking have potential drawbacks, including reactivity of active groups with host tissues, leachable small molecules, monomer or initiator, limited working time for injection prior to gelation, or the need for additional equipment, such as a uv source.

In comparison stimuli-sensitive, and specifically thermoresponsive, hydrogels do not have these drawbacks. However, they can be significantly more difficult to customize without skewing the hydrophilic/hydrophobic balance and altering the LCST. The lack of covalent crosslinking also tends to yield less mechanically stable materials. Temperature and pH change can be used to initiate a phase change in these polymers leading to gelation, independently, or used in conjunction with success (16, 19). Temperature-

sensitive polymers have been widely studied (20, 21). Some of these polymers, such as pluronic F127, have successfully been used for drug delivery, but have met with difficulty when used for cell encapsulation where the amphiphilic nature of the polymers can lead to membrane destabilization resulting in cell death (22).

PoligoGel is a novel degradable thermosensitive hydrogel manufactured by SamYang Corp. of Korea covered by United States Patent number 7,655,735. As described in the patent, it is based on multiple alternating blocks of PEG and PPO or PBO joined by degradable dicarboxilic linkages and has an LCST near physiological temperatures. The general chemical structure of PoligoGel is M-X –O-[PEO-Y-PEO-C(=O)-R-C(=O)-0]$_n$-PEO-Y-PEO-O-X-M where M is either H or an anion, Y is either PPO or PBO, R is $(CH_2)_m$ with m being an integer from 0 to 20, and n is an integer from 1-100. The structure of the variety of PoligoGel used for this experiment is given in Figure 4.1. Variations in the structure modify the LCST of the resulting solution. The LCST is also dependent on the concentration of the polymer in solution; a 15% gel will form near 20ºC, a 10% gel near 25ºC, and a 5% gel near 32ºC. In addition to the LCST being dependent on the concentration, the gel stiffness also varies, with lower concentrations yielding lower stiffnesses. The 5% gel at body temperature is a soft gel that will not flow, but does deform under its own weight. The delivery of multiple different small molecules has been achieved *in vivo* with the use of PoligoGel, including interferon-alpha over 5 days, human growth hormone over 6 days, and granulocyte stimulating factor over 8 days.

PoligoGel is nontoxic to multiple cell lines including rMSCs, human umbilical vein endothelial cells (HUVECs), and mouse colon carcinoma (CT-26), and in the case of rMSCs does not influence their pluripotency. Here it is shown to be a suitable material for the delivery of bone marrow-derived rat mesenchymal stem cells (rMSCs). Cells in the gel remain viable for over 7 days, are able to express protein when transfected while in the gel, and should escape the gel as it degrades. When used with transfected MSCs, excreted growth factors are able to diffuse from the gel and elicit a growth response in HUVECs, allowing for a wide range of potential treatments.

4.3 Materials and Methods

Rat mesenchymal stem cells were obtained from Tulane University's Center for Gene Therapy and expanded as recommended. In brief, cells were grown in Minimum Essential Media alpha (Invitrogen, Catalog number 12561-056), supplemented with 20% FBS (biowest, Catalog number S01520) and 1x Penicillin/Streptomyosin (Invitrogen, Catalog number 15140-122). Media was exchanged every 3-4 days. Cells for expansion were plated at approximately 100 cells/cm^2. Cells are passaged at a confluency of 70-90%. To lift the cells, they are washed in PBS and incubated in TrypLE™ Express (Invitrogen, Catalog number 12605-010) for 5 minutes, or until detached from the plate. CT-26 cells were grown in RPMI-1640, 10% FBS and 1x Penicillin/Streptomyosin, but otherwise cultured the same as the MSCs. HUVEC cells were grown in EGM-2 BulletKit media (Lonza CC-3162) used as supplied. Cells were passaged at

90% confluency, and media was refreshed every 2-3 days. Primary cells were used at passage numbers 10 or less.

MTT assays were performed by adding MTT to fresh growth media to a final concentration of 0.2mg/mL. Cells were incubated for 3-4 hours at 37°C 5% CO2 to allow the conversion of MTT to formazan crystals in the mitochondria of the viable cells. The media was then removed, and the cells were washed with PBS. The formazan crystals were then dissolved with DMSO and the absorbance was read on a plate reader (BioRad Model 680) at 570nm.

Trypan Blue 0.4% (Sigma, T-8154) was added to cells in suspension at a 1:1 ratio. The mixture was allowed to incubate at room temperature for 5 minutes, after which live and dead cells were counted on a hemocytometer.

Cell viability by alamarBlue was accomplished according to the manufactures instructions. Briefly, cells were plated in white clear bottom 96-well plates. At the required time points, 10x alamarBlue was added to the culture media and allowed to incubate for between 4 hours and overnight, depending on the cell concentration. Plates were then read on a PerkinElmer LS 55 Luminescence Spectrometer.

Fibrin gels were prepared in the following manner. Fibrin powder (Sigma, F2629-5g) was reconstituted in PBS by adding the fibrin to the top of the PBS in a 50ml tube with gentle rocking and allowed to dissolve. Fibrin concentrations were calculated to be near 10mg/ml using uv/vis. Thrombin (Sigma, T4648) was reconstituted in PBS at 100 units/ml and stored at -20°C

until used. Gels were made in 96-well plates by adding 20 uL thrombin to 30 uL fibrin solution.

The following procedure was followed for differentiation. Cells were grown to confluency in 6-well plates prior to adding the appropriate induction media. For osteogenic differentiation, it included complete media supplemented with 10mM β-glycerol phosphate, 50 μg/ml ascorbic acid, and 100nM dexamethazone; media was refreshed every 3-4 days. Adipogenic differentiation media was used as reported by Winer et al. (23). Induction media consisted of growth media supplemented with 1 μM dexamethasone, 200μM indomethacin, 10 μg/mL insulin, and 0.5mM 3-isobutyl-1-methylxanthine, which was placed on the cells for 3 days, followed by maintenance media (complete media with 10μg/ml insulin) for 1 day. This cycle was repeated twice, for a total of 8 days. All of the supplements used were obtained from Sigma, and were cell culture tested.

PoligoGel (SamYang Corp.) was obtained in a lyophilized cake. To hydrate the polymer, it was mixed with unsupplemented growth media or PBS and stirred overnight at 4°C to make a 10% w/v solution. For suspending cells in the gel, a 10% PoligoGel solution was mixed at 4 °C 1:1 with cells suspended in complete media to give a 5% polymer solution and the desired concentration of cells. The 5% solution was then placed in a cell culture incubator at 37 °C. 5% final solutions were used unless otherwise noted. Properties of 5% gels are mechanically sufficient for cell delivery without the added viscosity and associated difficulty of working with higher fraction gels.

For SEM analysis, samples were prepared by flash freezing in liquid nitrogen, followed by lyophilization. Samples were then imaged on a FEI Quanta 600 FEG.

RNA was isolated from rMSCs using TRIzol reagent (Invitrogen, 15596-018). cDNA was created with SuperScript III First-Strand (Invitrogen, 18080-051) using oligo DT primers following the recommended protocol. PCR for amplification of the genes of interest was performed using either Kapa2G Fast PCR Kit or the Hot Start kit (KapaBiosystems). Primers used for amplification are listed in Table 1.

Cells were grown in standard culture conditions to roughly 50% confluency. The cells were then transfected in serum free media and allowed to incubate for 4 hours, after which they were placed back in standard growth media or embedded in a 5% PoligoGel as described above. Cells were transfected with branched Poly(ethyleneimine) at an N:P ratio of 10:1.

4.4 Results

Viability of cell types tested was not affected by the presence of PoligoGel at any of the concentrations tested. PoligoGel was tested on rMSCs, CT-26 (mouse colon cancer) cells, and Human Umbilical Vein Endothelial Cells (HUVECs). In all cases, the same trend was observed: no toxicity at nongelling concentrations of 0.1%, 0.5%, and 1% to a 5% or 10% gel. In each case, there was no statistical difference by MTT assay at nongelling concentrations. At 5% or higher solutions, the cells under the gel did not proliferate, but remained

viable as measured by Trypan blue. When confluent cells that were no longer actively dividing were used, there was no significant difference between the control and experimental groups, as shown in Figure 4.2. Experimental groups were normalized to samples without the use of PoligoGel; three replicates were used for each group.

When suspended in PoligoGel, rMSC do not suffer any significant cell death. For these studies, cells were tripsonized, pelletted, resuspended in growth media, and placed on ice to lower the temperature. The cooled cell suspension was then mixed with PoligoGel, also cooled on ice, to achieve final concentrations of 100k, 200k, 500k, and 1 million cells per milliliter and polymer concentrations of 5%. 500uL of the gel/cell mixtures were then placed in a 24-well plate. After 48 hours, cells were removed from the gel by cooling the gel and adding sufficient media or PBS to lower the total concentration of polymer to prevent a gel from reforming. The mixture was then removed from the well and centrifuged at 400 rcf for 5 minutes to pellet the cells, which were resuspended in growth media and analyzed with trypan blue. The viabilities ranged from 95% at 100k per ml to 85% at 1 million per ml.

Cells embedded in PoligoGel show a spherical morphology with no visible protrusion into the gel when viewed by light microscopy, not the typical spindle shape seen when cultured on substrates such as fibrin with sites for cell binding. They also do not appear to proliferate based on the observation that cells remain suspended and are typically seen independent of other cells, not forming clusters as would be expected for dividing cells unable to migrate

through the substrate. Scanning electron microscopy confirms this, with cells appearing to be encased in the polymer, in stark contrast to cells grown in or on a fibrin gel where cells can be seen to attach and spread, resulting in a spindle shape as seen in Figure 4.3. Cells removed from the gel and plated in standard tissue culture dishes were able to resume proliferation, as seen when differentiating cells removed from the gel.

When culturing stem cells, others have observed that the substrate properties influence cell fate along different differentiative pathways (23). Substrate properties are also important for other cell types as well (24, 25). MSCs growing in PoligoGel are exposed to a soft three-dimensional scaffold; they then undergo a halt in proliferation, and an induced morphological change. It was possible that cellular capacity to differentiate was effected. To ensure that PoligoGel does not push rMSCs to differentiated fates, cells were cultured for 1 week suspended in the gel at about 100k cells/ml before being removed. Cells were then placed in differentiation media, or had the RNA immediately collected for PCR analysis following removal from the gel. Cells induced to an adipogenic fate were confirmed to develop into adipocytes by both the presence of lipid droplets in the cells (data not shown), as well as expression of genes indicative of an adipocyte phenotype. Control cells removed from the gel not placed in adipogenic induction media did not express the genetic markers for adipocytes, either immediately upon removal or at the time the induced cells were analyzed. While it would be unlikely for the soft gel substrate to induce differentiation of the cells to a hard tissue, the

retained capacity to do so does allow for the confirmation of the cells' retained pluripotency. When treated with osteogenic media, cells removed from PoligoGel changed from a spindle shaped morphology to a more cuboidal shape. Cells were also harvested for RNA collection at the end of the incubation period for PCR analysis. Cells were positive for both osteogenic markers.

Peroxisome proliferator activated receptor γ (PPAR- γ) and Adipocyte P2 (aP2) were measured for adipogenic differentiation, and for bone Osteoblastic specific factor-2 also known as Runx2 (OSF-2) and Osteocalcin (OCN). For each pair, the first gene is an early marker and the second gene is a later marker for differentiation along that path. The early markers may be constitutively expressed at low levels. Cells removed from PoligoGel did not exhibit either of the late differentiation markers, but there was some up regulation in the expression of PPAR-γ in the cells removed from the gel. Differentiated cells did express both RNAs with or without incubation in the gel. PCR results looking for the different marker genes are shown in Figure 4.4.

PoligoGel was also tested for its effects on protein expression in transfected cells. MSCs were transfected with a luciferase encoding plasmid and 4 hours posttransfection were removed and placed in PoligoGel, or replated in a standard cell culture environment. Luciferase expression was observed at 2 and 4 days posttransfection. Cells were also allowed to persist in PoligoGel for 2 days, and were then removed from the gel and placed in a standard tissue culture dish. No difference was seen between cells within, or without PoligoGel, as seen in Figure 4.5.

The ability of transfected rMSCs to elicit a growth response was tested on HUVECs. As previously mentioned, cells grown under gelled PoligoGel did not proliferate. To avoid this effect, HUVECs were plated in 96 well plates, and 50 uL of a fibrin gel was placed on top of the cells. MSC growth media was added to the wells after the fibrin had form a gel, and allowed to sit overnight. The following day, the excess media was removed by inverting the plate over sterile gauze, allowing the media to drain by gravity. Transfected MSCs were mixed in PoligoGel 4 hours posttransfection, and 200 uL of the gel were added to each well. The cell concentration was approximately 100-200k cells/mL. After 48 hours for cell growth, the plates were placed at 4 degrees C for 5 minutes and had 200mL cold PBS added. The MSCs and excess PoligoGel were then removed by inverting the culture plate over sterile gauze. 200 uL of growth media were then added, above the fibrin gel and HUVEC cells. Alamar blue was then used to assay the number of cells in each well. As seen in Figure 4.6, all groups outperformed the HUVEC only control, and all growth factor transfected groups outperformed the addition of unaltered stem cells.

The persistence of the transfection was observed with PCR and is shown in Figure 4.7. All transfections showed elevated mRNA levels of gene expression beginning at 4 hours and continuing to 3 days, with higher levels observed for FGF and VEGF at 7 days as well. Also of note is that the initial FGF transfection seems to have promoted an increase in the transcription of VEGF, and transfection with any of the three growth factors, but not a luciferase control, increased FGF mRNA levels. VEGF-transfected cells

continued to show increased FGF mRNA at 3 days out as well. By day 7, the expression levels had dropped back down near the levels of the control cells.

4.5 Discussion

Cell treatments will never reach their full potential if the cells fail to persist at the intended target. Cells are lost to washout from the injected site, migration, and cell death. Here it has been demonstrated that the use of a thermoresponsive hydrogel has the potential to be used for the *in vivo* delivery of cells. The use of the gel can reduce the loss of cells from the implant site due to washout and migration by forming a hydrogel scaffold that will hold cells near the injection site where they will be able to affect the desired tissues, both directly as the gel degrades and cells are able to escape, and indirectly by releasing growth factors both constitutively or by means of transfection. PoligoGel can be used to potentially reduce the number of cells required for injection by keeping a greater proportion of the cells nearby. While MSCs derived from bone marrow are relatively plentiful and easy to obtain, this benefit should not be over looked, especially for allografts of cells such as cardiomyoblasts, where yields are lower and more difficult to obtain (26).

PoligoGel was also shown to be nontoxic at low concentrations to multiple cell types although proliferation was adversely affected when a gel was formed above the cells. Mesenchymal stem cells suspended in the gel suffered no significant adverse consequences at cell concentration up to 1

million cells per ml. Cell viability remained high, above 90%, and dropped to 84% at the higher concentration. This cell death is not assumed to be due to PoligoGel or the encapsulation process, but rather due to lack of diffusion of oxygen and metabolites for cells farther from the surface resulting in necrosis, but this was not tested. Cells also do not appear to proliferate in the gel, based on the observation that cells remain suspended and are typically seen independent of other cells, not forming clusters as would be expected for dividing cells unable to migrate through the substrate. Studies using direct cell transplantations have used far greater numbers of cells for the injection, typically in the tens to hundreds of millions in humans(27). This would require far greater cell loading than would be practical with this system, but with the cells fixed in position, it is possible to have equivalent if not greater numbers of cells near the requisite site.

Maintaining viable cells is only part of the difficulty in using stem cells. The cells must retain their differentiative capacity in order to achieve the full therapeutic potential. PoligoGel does not have any observed effect on mesenchymal stem cells' ability to differentiate after 1 week of being exposed to the gel, either by pushing the cells to a differentiated fate, or limiting their ability to become terminally differentiated cell types. This allows for local cellular signals at the implant site to push the stem cells to differentiate into suitable cell types for wound healing, whether that is endothelial cell or smooth muscle cell for revascularization, or cardiomyocytes for replacing cells which may have died during the ischemic event.

Transfected cells can improve the outcome by delivering growth factors that can recruit additional cells to the tissues and also promote survival of the native cells. The use of PoligoGel does not impair the effectiveness of the transfection. Transfected cells suspended in the gel were able to elicit a growth response from HUVECs; a luciferase assay further confirmed no statistical difference in protein production between cells in the gel and those on standard surfaces (data not shown). Looking at mRNA expression demonstrated a typical expression profile with a peak at 3 days, and expression levels returning to control after 7 days. While the mRNA levels are not indicative of the levels of protein expression, it does indicate that the plasmids are active, and the translation is occurring. While both FGF and VEGF appear to be stimulating the production of the other growth factor, the growth factor used in transfection showed the highest expression. Alone or included with PoligoGel, none of the growth factors demonstrated any superiority over the others in regards to differentiative capacity of the cells; regardless of treatment, all the cells retained the ability to differentiate into adipocytes, or osteoblasts. While looking at HUVEC proliferation, there was no statistical difference between the different growth factors; PDGF-CC did show the highest proliferation. This in combination with the data from the previous chapter showing PDGF-CC and VEGF as having the highest angiogenic potential demonstrates the viability of using PDGF-CC as an appropriate choice for transfection of the MSCs to be implanted in the heart.

4.6 Conclusions

With the growing number of cell therapies being tested and developed, it is important to ensure that the cells are being used to the greatest advantage. The thermoreversible polymer PoligoGel has been shown to have the potential to be used for the delivery of stem cells. It does not influence stem cell differentiation and permits cell survival past 1 week when cells are imbedded in the gel. Despite spherical morphologies when in the gel, transfected cells embedded in the gel have been shown to be able to produce therapeutic proteins after transfection. Protein from transfected MSCs can be delivered from the gel, eliciting a growth response in other cell types. The use of this material has the potential to improve current cell treatments by retaining the cells at the site of implantation. As previously mentioned, a majority of the cells injected in saline are lost in as little as 30 minutes (5). The use of the scaffold will allow the expressed proteins to act at the implant site, as opposed to tissues where they eventually may engraft.

4.7 References

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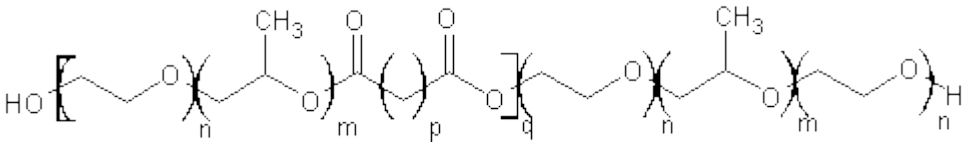

Figure 4.1 Representative structure of PoligoGel. PoligoGel is composed of alternating blocks of poly(ethylene oxide) and poly(propylene oxide) joined by ester bonds with a small hydrocarbon chain. For the gel used, the average values for the repeat units were n = 101, m = 56, p = 2, and q = 7.

Table 4.1: List of primers used in PCR for amplification of target genes.

Gene	Forward Primer	Reverse Primer
aP2	AAT TCG GCA CGA CTC CTT GAA AGC	TGG TCG ACT TTC CAT CCC ACT TCT
PPAR-γ	TCT CCA GCA TTT CTG CTC CAC ACT	ATA CAA ATG CTT TGC CAG GGC TCG
Runx2	CAA GTG CGG TGC AAA CTT TCT CCA	TGT TTG ACG CCA TAG TCC CTC CTT
Osteocalsin	AAT GCC ACT GCG TAT TGG TTG ACG	TGG CGG TGA GAT AAT GGA TGT GGT
GADPH	GAC CCC TTC ATT GAC CTC AAC TAC	AGA TGA TGA CCC TTT TGG CTC C
VEGF	GCC AGC ACA TAG GAG AGA TGA G	GCT TGT CAC ATC TGCA AGTA CG
FGF	CAA GCA GAA GAG AGA GGA GTT GTG TC	TCA GCT CTT AGC AGA CAT TGG AAG
PDGF-C	TCC TGC TGA CAT CTG CCC TG	CAG GAG ACA ACC TGG CCA G

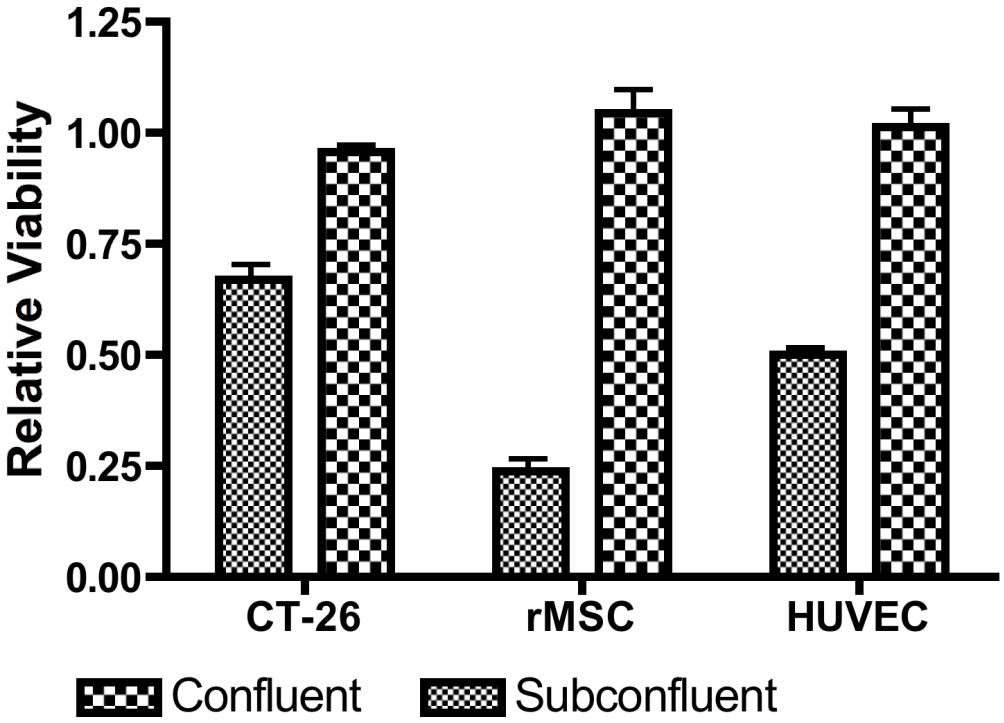

Figure 4.2: While the gel does not induce cell death, as confirmed by trypan blue staining, it does limit proliferation. When placed above confluent cells, there is no change against confluent controls. Variations in the subconfluent values are based on differences in the initial number of cells present. Error bars are the standard deviation, n=3.

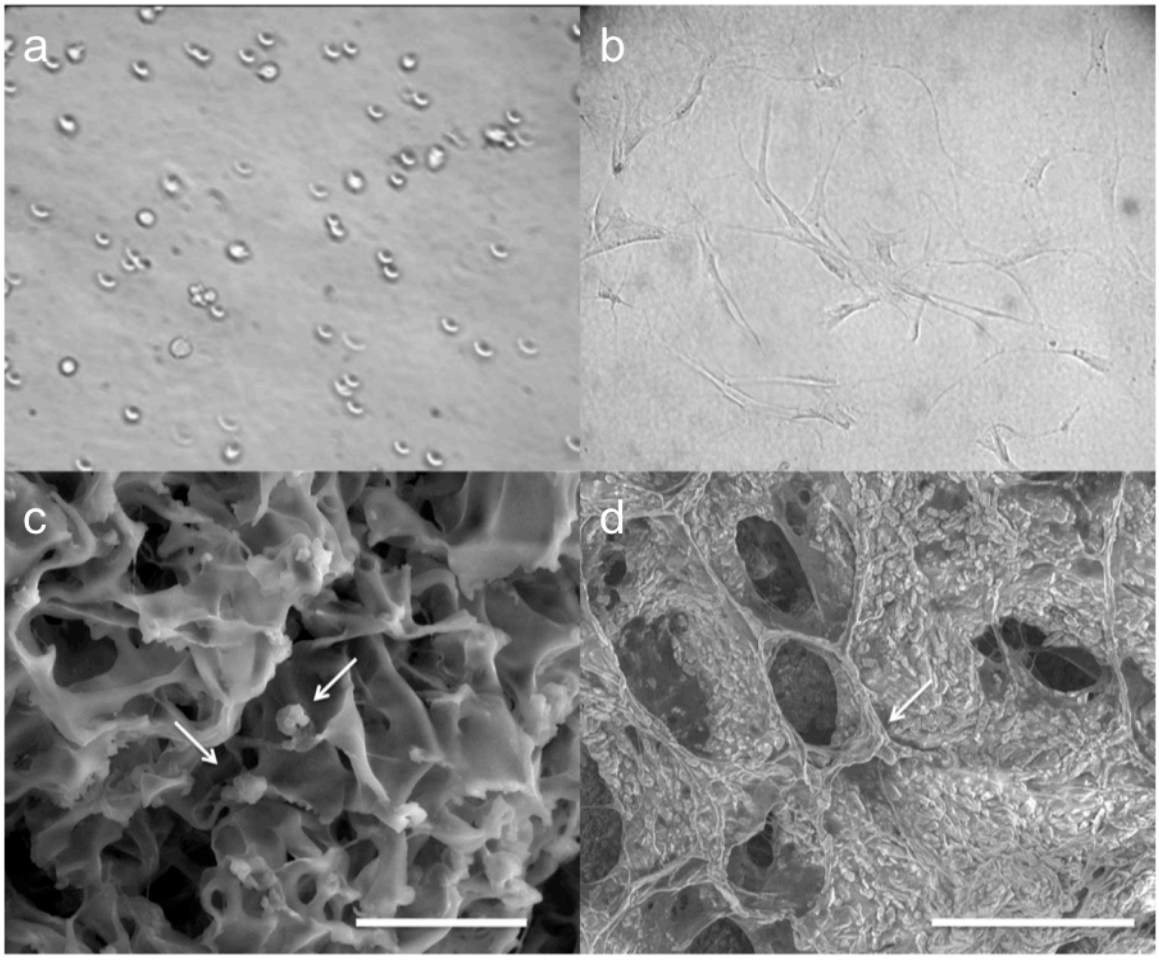

Figure 4.3: Images of rMSCs on PoligoGel and Fibrin Gels. a) Image taken with a light microscope with cells in PoligoGel 5%, b) light microscope image of rMSCs growing on a fibrin gel, c) SEM image of rMSCs embedded in PoligoGel, d) fibrin gel with rMSCs. While the fibrin promotes cell attachment and spreading, no evidence is seen of cell attachment in the PoligoGel samples. Arrows indicate the location of cells in the corresponding matrix. Scale bars are 50 μm in the SEM images.

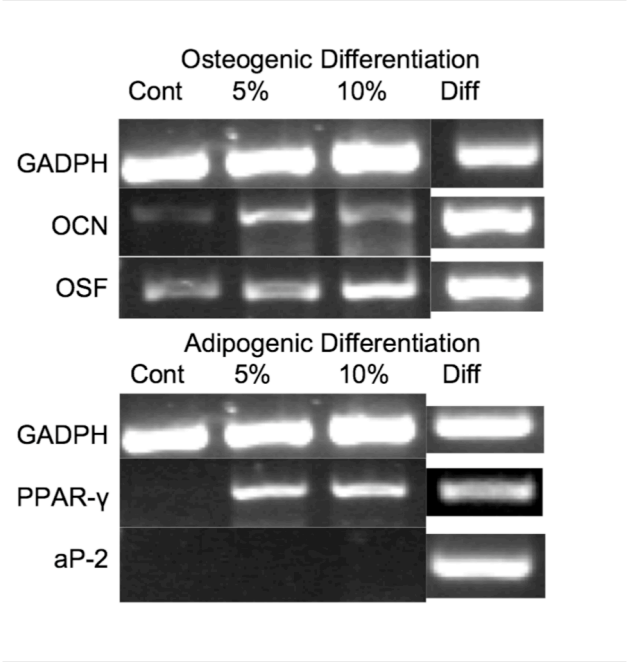

Figure 4.4: MSC differentiation did not occur as a result of exposure to PoligoGel at either a 5% or 10% concentration, although they did express low levels of some factors compared to control groups. Cont – Control cells grown on standard tissue culture surfaces; 5% - Cells grown in 5% PoligoGel; 10% - Cells grown in 10% PoligoGel; Diff – Control cells exposed to differentiation media without prior exposure to PoligoGel. Cells removed from PoligoGel retained the ability to differentiate, as evidenced by production of both markers.

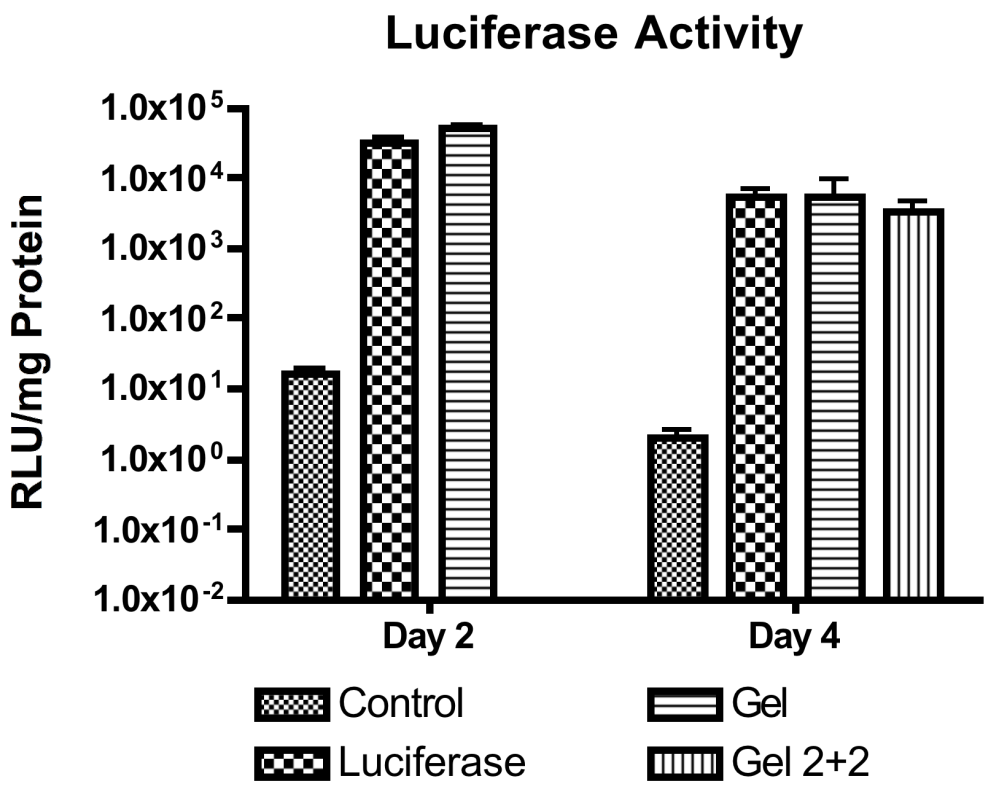

Figure 4.5 Transfected cells were placed in PoligoGel (Gel), or replated (Luciferase) after transfection with a luciferase encoding plasmid. Gel 2+2 indicates cells that were first placed in PoligoGel for 2 days, and then grown in standard conditions for the following 2 days. There was no difference between the groups on the same days. Error bars are the standard deviation, n=3.

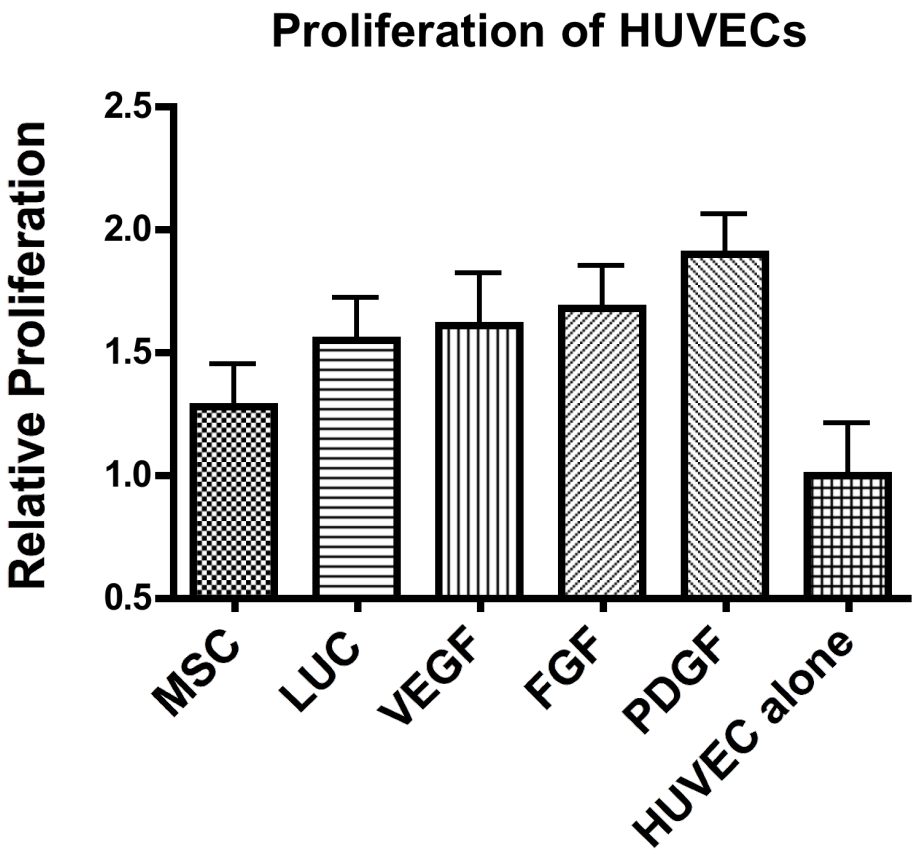

Figure 4.6: All transfected groups were significantly improved over HUVEC only group ($p < 0.05$), which was given a value of 1 for comparison to the other groups. PDGF-C was significantly different from all nongrowth factor sets at $p < 0.05$, the difference was not significant between the growth factor transfected groups. Data are the average of 6 wells; error bars are the standard deviation. Data were analyzed by ANOVA with Bonferroni posttests to test differences between groups.

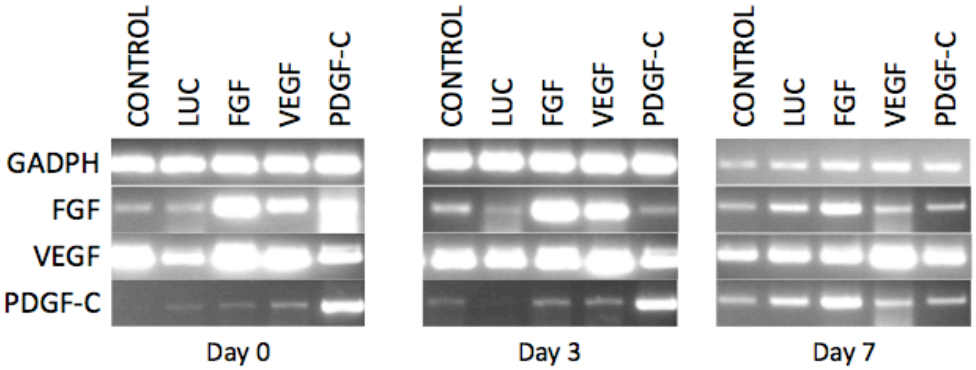

Figure 4.7: PCR data showing the expression of various growth factors both constitutively and as a result of transfection. Rows are genes of interest, and columns show the gene with which the cells were transfected if applicable. Elevated mRNA levels were seen for both FGF and VEGF out to 7 days, with both of them positively influencing the expression of the other.

CHAPTER 5

IN VIVO STUDY OF EFFICACY OF TRANSFECTED MSCS FOR THE TREATMENT OF CARDIAC INFARCT IN A RAT MODEL

5.1 Abstract

Heart failure is marked by the loss of viable tissue and the formation of scar tissue at the site of impaired blood flow. This scarring results in negative remodeling of the heart to achieve the same ejection volume, while equalizing the stresses in the heart. This results in ventricle dilation and hypertrophy of the still viable muscle. Mesenchymal stem cells can be used as a therapeutic device in treating heart disease. Cell delivery is impaired by the loss of cells at the injection site. A tissue scaffold has been used to retain cells at the heart, to allow for a longer, more effective treatment window. MSCs can help foster angiogenesis, helping to restore lost blood flow. In addition to the cells integration into the myocardium, they have also been transfected to express PDGF-CC. PDGF-C is an angiogenic factor, stimulates the release of VEGF, and helps contribute to mature newly formed blood vessels.

Testing of the proposed treatment was accomplished in a rat coronary infarct model. The heart was accessed via thoracotomy, after which the left coronary artery was ligated. The treatments were given shortly after the ligation. Animals were analyzed by cineMRI to observe the heart function. The biodistribution of the cells was also examined by PCR; test animals were all female, and the injected cells were from male rats, allowing for the measurement of genes associated with the y-chromosome.

There was no significance observed in the MRI data, for ejection fraction, wall thickness, heart volume, or stroke volume. Biodistribution showed an increase of over 15 times more cells in heart for groups where the cells were delivered with a scaffold.

5.2 Introduction

The prevalence of cardiovascular disease has necessitated significant research into new treatments for recovery after ischemic events. One of the major limitations in treating patients who have suffered a heart attack is the lack of regeneration of cardiac tissue. This results in remodeling of the heart to retain the same ejection volume as prior to the infarct, and equalizing stresses in the wall of the heart (1-3). Many of the newest treatments focus on repopulation of the myocardium and preservation of cells in the ischemic region to minimize tissue loss. Repopulation of the heart has been attempted by cell injection (4-6), cell sheets (7), as well as recruitment of cells to the infarct site (8). Cells injected in saline have a low engraftment rate, and most

end up at sites away from their needed location. Cell sheets do not suffer from this limitation, but do require significantly more time and equipment for their creation, if autografts are used. Neither cell sheet autografts, nor relying on the recruitment of native cells to the myocardium, would be of use in the initial period of wall thinning and dilation of the ventricle, which occur in the initial 72 hours (3).

In addition to repopulation, effort is directed to the prevention of cardiomyocyte death. Many of the gene therapies for treating heart ischemia have focused on the delivery of anti-apoptotic cytokines, many of which are also angiogenic, or by the silencing of apoptotic factors (9). Gene delivery to the myocardium has focused on several different targets based in part on differences in the time from the ischemic event. In treating the heart shortly after the ischemic event, most of the work has been focused on saving tissue and the restoration of adequate blood flow. Some of the most common genes used are growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and platelet derived growth factor (PDGF). These genes are typically delivered directly to the heart to transfect local cells by means of intramyocardial injections, catheter-based perfusion, ultrasound targeted microbubble destruction, or retroinfusion (10). Injecting therapeutic plasmid DNA has been shown to be effective as a treatment for myocardial infarction. When used in a rabbit model where the circumflex artery was ligated to produce an infarct in the heart, the untreated animals developed an infarct of nearly half of the

ventricle. Animals treated with a VEGF plasmid delivered by a cationic polymer developed an infarct of roughly 15% of the left ventricle (11).

Transfected cells used to deliver growth factors to the myocardium have also yielded promising results. Myoblasts transfected with VEGF encoding plasmid restored function to the heart following transplantation into the ischemic myocardium. This was measured by observing the ejection fraction, wall thickness, and end systolic and diastolic volumes (12). The use of MSCs with VEGF has also shown to be myogenic and prevent the progression of heart dysfunction (13).

Genes are also being targeted for silencing. Shutting off of correct genes can lead to increased survival of myocardial tissue in the ischemic region. While growth factors often have some anti-apoptotic effects, other proteins such as Bcl-2 can be much more potent at achieving cell survival (14). Hif-1a is a transcription factor which when expressed at higher levels can lead to increased expression of many genes, including genes regulating apoptosis and resistance to oxidative stress (15).

Scaffolds have also been used, and shown to have beneficial effects on the ischemic heart (16, 17). The combined use of transfected stem cells retained at the heart by the use of an *in situ* forming hydrogel has the potential for improving the efficacy of treating myocardial infarction, as is shown here in a rat model.

There are multiple factors that can be analyzed to study heart function. Those observed here by MRI are the ejection fraction, wall thickness, and heart

volumes. Ejection fraction is the measurement of the fraction of the blood that is pumped out of the heart with each beat; it is a measurement of how efficiently the heart is working. Wall thickness can be used to observe scar formation as well as remodeling of the heart. The heart volume is also a measure of the remodeling that has occurred in the heart.

5.3 Materials and Methods

5.3.1 Cell Culture

Rat mesenchymal stem cells were grown in Minimum Essential Media alpha (Invitrogen, Catalog number 12561-056), supplemented with 20% FBS (biowest, Catalog number S01520) and 1x Penicillin/Streptomyosin (Invitrogen, Catalog number 15140-122). Media was exchanged every 3-4 days. Cells for expansion were plated at approximately 100 cells/cm^2. Cells are passaged at a confluency of 70-90%. To lift the cells, they are washed in PBS and incubated in TrypLE™ Express (Invitrogen, Catalog number 12605-010) for 5 minutes, or until detached from the plate.

PoligoGel was mixed in MEM – alpha overnight at 4 degrees for a final concentration of 10% W/V, and sterile filtered prior to use. Cells were suspended in MEM-alpha at a concentration of 2 million/mL and subsequently mixed with 10% PoligoGel, giving final concentrations of 1 million cells per mL, and 5% PoligoGel. Cell/gel mixes were loaded into syringes, and stored at 4 degrees in a refrigerator until use in the animals. Transfection was performed on cells at about 50% confluency in serum free media and allowed to incubate

for 4 hours, after which they were placed back in standard growth media or gel mixed with growth media. Cells were transfected with branched Poly(ethyleneimine) at an N:P ratio of 10:1. Cells were used for surgery 24 hours after transfection.

5.3.2 Animal Model

The following materials were required for animal surgeries: 16 g (BD381457) 1¾ in for endotracheal tube, guide wire, surgical tape (3M 1527-1), syringe (TB, 1, 0.5cc), alcohol wipes, needle (30g), providone-iodine wipes, 10 x 10 cm^2 gauze, 5 x 5 cm^2 gauze, EKG machine with EKG electrode needles (3), pulse oximeter, scale, animal hair clipper, ventilator, small scissors, 0 silk suture for tying the endotracheal tube around the neck, optical fiber light source, spatula, intubation board, ear punch, hair cap, sterile gloves, and surgical masks.

The sterile instrument tray included one of the following unless noted differently: finger-loop locking (Wheatlander) retractor, scalpel holder for #10 blade, needle driver, hemostats (2), long fine forceps, dish for saline, cutting scissors, dissecting scissors (Metzenbaum), 10 x 10 cm^2 gauze (4-5), 5 x 5 cm^2 gauze (4-5), cotton tip applicator (10), blue towel (2). These additional accessories were also used: 3.0 vicryl suture (1 per rat), 6.0 prolene suture (½ per rat, for ligation), normal saline (for the dish), #10 surgical blade, 30g needle (1 per rat, for injection only) (BD305106), TB syringe (BD309602).

Medications for the animals included Ketamine, Atropine, Cefazolin, and Buprenorphine. Ketamine was dosed at 44mg/Kg and injecting intramuscularly. Atropine was dosed at 0.02-0.05mg/kg and injected intramuscularly. Cefazolin was reconstituted at 0.1g/ml and 0.25 ml was administered by intraperitoneal injection. Buprenorphine at 0.3mg/mL was administered intramuscularly at 0.01-0.05 mg/Kg. Lidocane (2%) was used to numb the throat prior to intubation.

The rat infarct model was executed in the following manner. Female Lewis rats weighing 190-220 g were anesthetized in an induction chamber with isoflurane at 4%. Once the animal was asleep, ketamine was injected into the right thigh, the ear was punched for identification, and the hair was shaved from the left anteriolateral chest using animal clippers. The animal was then returned to the induction chamber with oxygen only to awake; 10 minutes were allowed to pass to ensure the ketamine had taken effect. Isoflurane (4%) is again used to sedate the animal prior to intubation. Once asleep, the rat is given the proper doses of both atropine and cefazolin.

Intubation was performed in the following manner. The IV catheter had a small section of tubing slid up the outside of the catheter to a distance of about 1cm from the end. The guide wire was then inserted through the catheter. The rat was hung by the incisors on an intubation board. The tongue was drawn from the mouth using the cotton end of an applicator, and held out of the mouth with the other hand. The stick end of applicator was then used to hold the tongue out of the way, and the mouth open. A second applicator

was used to apply lidocaine to the throat and vocal cords and massage them open. The guide wire was inserted into the larynx, the catheter slid down the wire into place and the guide wire removed. Proper placement was checked for using a clean polished spatula to observe condensation from the exhaled breath.

Once proper placement of the endotracheal tube is obtained, the animal is transferred to the surgery table, carefully holding the tube in place. After checking the proper placement of the endotracheal tube again, 0 silk suture is used to tie the catheter around the head to prevent movement. The animal is then hooked to the ventilator and the ventilator is started with 3% isoflurane.

The left arm of the rat is taped above the head, the pulse oximeter and EKG are attached and signals obtained. The shaved portion of the chest is cleaned with the providine-iodine wipes.

The technique used for the operations is descrbed here. Once in sterile surgical gear, a blue towel is draped over the rat and a small 4x4 cm hole is cut in the towel, exposing the shaved area on the animal. The heartbeat is felt through the chest wall, and using the scalpel, an incision is made at the inferior location where the heart is felt strongest. Using the scissors, the muscle layers were cut down to the rib cage. Once to the ribs, the lower intercostal space where the heartbeat can best be felt is entered. After carefully entering the thoracic cavity, the intercostal space is spread with the retractors.

A small portion of a gauze sponge is moistened and used to sweep the lung out of the way. The pericardium is then cut away from the heart. After finding the left anterior descending artery, it is ligated with a single stitch using 6.0 Prolene suture. If the infarct size was insufficient a second stitch was used closer to the aorta. The appropriate treatment was then applied to the infarct zone. After removing the gauze, the chest is sewn closed in three layers using 3.0 Vicryl. First, the ribs are closed using a figure-of-eight suture. Second the muscle layers are closed with a running stitch. Finally, the skin is closed using a running subcuticular stitch. Once the rat was breathing on its own, and the blood oxygenation was stable above 75%, the animal was moved to a cage under a heat lamp.

5.3.3 MRI Analysis

CINE MRI analysis was performed on a Bruker Biospec 7T/30cm system using Bruker AVANCE II electronics. Transmission and reception were achieved using a Bruker birdcage quadrature resonator with a 72 mm inner diameter.

Animals were imaged 4 weeks postsurgery. Animals undergoing imaging were anesthetized using isoflurane, 4% for induction, and maintenance at 2.5%. Rats were monitored using a pulse-oximeter attached to the hind paw, and a pressure sensor to monitor respiration. Body temperature was monitored by a rectal thermometer, and controlled by a warm air heater.

Gating for the images was accomplished by the use of IntraGate software package from Bruker. Slices were taken from the apex of the heart to

the top of the ventricles in 1.5 mm slices with 15 time increments dividing a single heartbeat. The resolution of a single frame was 0.26 mm on the x-axis, and 0.15 mm along the y-axis based on the size of the pixels. An example image is given in Figure 5.1, a slice taken from a rat receiving cells in PoligoGel as a treatment. Images are shown for both the native image, as well as with contours drawn which were used for analysis. Analysis of the resultant images was accomplished using the freely available software packages Segment v1.8. The left ventricle was analyzed for ejection fraction, wall thickness, and ventricular volume.

5.3.4 Biodistribution

Biodistribution is being performed by RT-PCR. All the animals used are female, and the cells being injected are from male rats. This allows for cells to be tracked in the animal by looking for genes specific to the 'Y'-chromosome, the sex-linked region 'Y'. Multiple controls will be used in the analysis, to minimize differences in expression between tissues. Genes to be used for controls include beta actin, cyclin-dependent kinase inhibitor 1B (CDK1b), cyclin-dependent kinase inhibitor 1A (CDK1a), hypoxanthine phosphoribosyltransferase 1 (HPRT1), E74-like factor 1, and eukaryotic translation initiation factor 2B, subunit 1 alpha. The control genes were amplified using master mixes obtained from Applied Biosystems (Carlsbad California) and used as purchased, catalog numbers Rn00667869_m1, Rn00582195_m1, Rn00589996_m1, Rn01527840_m1, Rn00585356_m1, and

Rn00596951_m1, respectively. Primers and probe for the SRY sequence were purchased from Applied Biosystems and designed using the Primer Express software program using accession number NM_012772.1. The primers used were GGC GCA AGT TGG CTC AA for the forward primer, GGC AAC TTC ACG CTG CAA for the reverse primer, and AGA ATC CCA GCA TGC AGA was used for the Taqman probe. PCR was performed using TaqMan® RNA-to-CT™ 1-Step Kit, part number 4392938, on an Applied Biosystems StepOnePlus machine. RNA was loaded at 100 ng per well.

Tissues were harvested after 24 hours. Rats were sacrificed by isoflurane overdose after an injection of heparin. The abdominal wall was opened and the aorta severed. As the organs of interest were removed, they were immediately frozen in liquid nitrogen. Tissues were stored at -70ºC until homogenized with a mortar and pestle. In the case of the heart, the tissue was thawed with RNAlater-ICE (Applied Biosystems, AM7030) for removal of the left ventricle, then homogenized.

5.4 Results

A total of 34 animals were used for the MRI analysis. Some animals had insufficient infarcts to be included in experimental groups. Ejection fraction was used as the excluding factor; animals with an ejection fraction over 70% were not used for analysis in the experimental groups where an infarct was presumed to have been caused. All groups had 5-6 animals in the set.

5.4.1 MRI Analysis Results

After successful application of the coronary ligation model, imaging was performed at 4 weeks (an average of 29 days) postinfarct. The resulting scans were then analyzed using Segment software. The ejection fractions for the rats are given in Figure 5.2. The average ejection fraction for the non-infarcted heart was 78.1% ± 1.4. The ligation only group had an average ejection fraction of 45.9% ± 14.2. Transfected cells had an average of 52.1% ± 9.4. PoligoGel alone showed an average of 51.0% ± 15.5. PoligoGel with untransfected stem cells had an average ejection fraction of 50.9% ± 14.1. The PoligoGel with transfected stem cells yielded an average of 47.6% ± 11.7. There was no statistical difference between any of the infarcted groups based on a one-way ANOVA analysis.

Wall thickness was also calculated based on the MRI images. The thickness was calculated by the Segment Software package after manually tracing the surfaces of the heart. The thickness was calculated across 8 segments of the myocardium. Segments 1-3 were typically healthy tissue. Segments 4-6 included the septum. Segments 7 and 8 typically were composed of the infracted zone with segment 8 being more fully infarcted tissue. This measurement was more challenging to perform due to poorer quality of the epicardial surfaces compared to the endocardial surfaces at some areas of the heart. A typical thickness for a healthy heart is around 3mm; an infarcted tissue can lose 1-2 mm in thickness (12). The results are given for both systolic and diastolic thicknesses in Figure 5.3. Viable, contractile heart tissue will

thicken as a result of the contraction, while scar tissue will have no such change in morphology. The lack of thickening can be seen in Figure 5.4 where the change in thickness for sector 8, the predominately infarcted sector, is given. There is little contractibility in the infarcted hearts, but no significant difference between the treatment groups. The final parameter gained from the MRI analysis of the hearts was the heart's end systolic and diastolic volumes. Figure 5.5 shows the volumes for the various treatments. There were again no significant differences. The stroke volumes, however, did show differences, with all groups different from the thoracotomy alone, as seen in Figure 5.6.

5.4.2 Biodistribution

Of the many different potential controls evaluated for use, beta actin, CDK1b, and HPRT1 were found to be the most consistent in achieving proper amplification across the range of tissues.

Tissues examined included the left ventricle, the remainder of the heart, kidney, spleen, bone marrow, liver, and lung. Cell numbers were approximated by mixing cells with processed heart tissue in known amounts corresponding to 1 million cells per gram of heart tissue, down to 100 cells per gram of heart tissue and then generating a regression curve from the results.

Real-time PCR showed that cells delivered in PoligoGel were retained at a significantly higher level than those injected without the gel. The left ventricle showed almost 850,000 cells per gram of tissue for cells injected with PoligoGel while the cells alone were retained at a rate of about 55,000 cells

per gram. The results were significant with $p < 0.001$. A slight number of cells were found in the lung of one of the animals with no PoligoGel used, but there was no other definitive discovery of cells in any of the other tissues with either set.

5.5 Discussion

The variance in the infarct size prevented statistical significance from being realized in the MRI data despite others having used this model as a viable method of testing i*n vivo*. There are several possible reasons for this discrepancy. First, while performing the procedures on the animals, greater precision in the placement of the ligating suture would have been beneficial. Ligation was typically performed where the artery was most visible, and not based on landmarks on the heart, such as the aorta. The slight variations in the anatomy of the hearts of individual rats and slight variations in the surgical entry point which led to a lack of uniformity in the ligation site could have been ameliorated by additional practice, especially with one more familiar with the procedure. Even with the surgeries as performed, this lack of consistency might have been alleviated with multiple measures of each animal and analyzing the data with a repeat measures ANOVA looking for the improvement in the hearts, or the lessening of the hearts deterioration. Larger groups of animals would also have helped to acquire lower standard deviations, potentially showing significance where the smaller groups failed. Larger

groups, as well repeated measures, gets much more expensive, due to the additional MRI scans required, and the additional animals in larger groups.

In addition to the alternatives presented above, a different model could have been used, as an example, that of a cryoinjury model. Here a probe, typically exposed to liquid nitrogen, is used to freeze and kill the tissue. This results in a very reproducible sized defect determined by the size of the probe and the duration of contact with the heart. It is, however, a less accurate model in looking at cell survival. Cells in the heart defect die as a result of the freezing, not as a result of blood flow. This makes it impossible to view increases in cell survival by staving off apoptosis, or necrosis as a result of nutrient depletion and waste accumulation. It can be used for looking at remodeling of the heart, as well as neovascularisation.

While not directly related to the results, it is possible that a longer interval between treatment and data collection should have been used. Remodeling of the heart after an infarct is typically seen as two end results. First is the redistribution of stresses in the heart as a result of the lost tissue and contractility. Second is dilation of the ventricle resulting in the same stroke volume even at the lower ejection fraction (2). Stresses in the heart were not analyzed, but based on the stroke volume data, 4 weeks was insufficient for full remodeling. Noninfarcted controls showed average ejection volumes of 0.12±0.04 mL; treated animals showed ejection volumes ranging from 0.06±0.01 to 0.09±0.01 mL with a positive correlation between ejection fraction and ejection volume.

The results of the biodistribution showed that the PoligoGel performed as expected in retaining the cells at the site of the injection, with 15 times the number of cells retained, based on the experimental results. With the improved retention, it is dissapointing that no benefit to the hearts' function was seen, concidering that in other studies cells alone have shown improvement the heart function (18). The inclusion of a different growth factor, such as FGF or VEGF, should have made a more significant difference on the results. The lack of improvement after such success in retaining the cells is puzzling, but there are several possible explanations. First, although the cells were retained in greater numbers at 24 hours, it is possible that at longer time points, cells were lost at equal numbers. This could occur as a result of cells that are released from the gel as it degrades being lost from the heart in a fashion similar to that of cells injected without the scaffold. The PoligoGel could even exacerbate this, as it has been shown to impede cell adhesion *in vitro*. This same effect of impaired cell adhesion could also lead to poorer chemotaxis of the animal's own cells to the site of the infarct. While there were no data collected to support either of these possibilities, none was collected to refute it either; and neither would explain why the growth factors produced by the stem cells were not more effective. Gene vectors for VEGF injected into the heart were shown to be effective in some studies (19, 20); others studies did not show therapeutic results (21). There are two main differences between the VEGF studies and that presented here. First, the growth factor of choice here is different. PDGF-C has not been known as long,

or studied as well as other growth factors, such as VEGF, or FGF. Despite promising results prior to the animal work, it may have been a poor choice, due to lower cellular activation, higher effective doses required, or other reasons. Secondly, different populations of cells were transfected, and presumably different numbers of transfected cells were resident in the heart after the procedure. While the growth factors produced by either population should be identical, the rate of production between individual cells of different origins can vary. MSCs were shown to have a transfection rate of about 10%, leading to an estimate of 30,000 transfected cells being injected to the heart. Direct injection of a gene vector into the heart could potentially yield a higher number of transfected cells, leading to the improved outcomes. If the number of transfected cells is the limiting factor, it could be overcome by cell sorting. Dual encoding plasmids, which express both the therapeutic gene, as well as a marker, such as GFP, can be used for the transfection. Flow cytometry can then be used to isolate transfected cells, allowing for the injection of a fully transfected population.

5.6 Conclusions

As established in the introduction, the use of stem cell and growth factors for the treatment of infarcts has shown potential in multiple instances, both individually, as well as combined. This potential was not realized here in the treatment of the infarcts. Despite a lack of improvement in the heart,

PoligoGel did work as intended in retaining cells in the injection site, at least in the short term.

Several changes should be made to better ascertain the true potential of using a scaffold for injecting cells: first, an improvement in the surgical reproducibility; second, modified analysis, either by increased sample size, or taking repeated measurements; third, working with a growth factor previously proven to improve heart function. These changes should permit the acquisition of data showing the improved outcome with the use of cells injected in a thermoreversible polymer scaffold.

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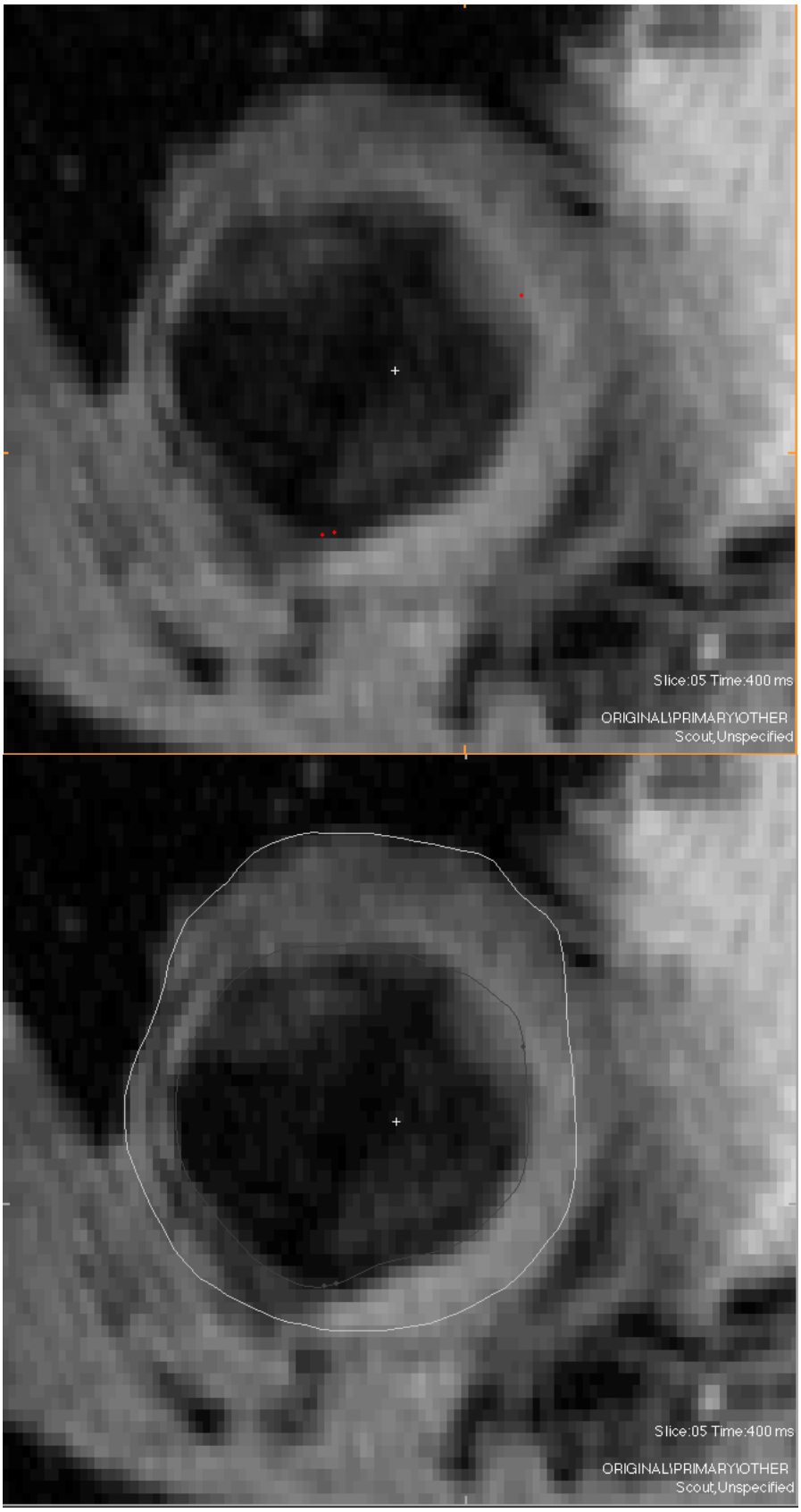
Figure 5.1 Example images of MRI data taken from a rat receiving cells in PoligoGel as a treatment. Slice shown is from about 6 mm above the apex of the heart. Pixel size for the images was about 0.15 mm along the x-axis, and 0.26 mm along the y-axis. The top image shows the raw images, while the bottom has had the contours of the inner and outer walls of the ventricle traced in a dark and light line, respectively.


Slice:05 Time:400 ms
ORIGINAL\PRIMARY\OTHER
Scout, Unspecified
Slice:05 Time:400 ms
ORIGINAL\PRIMARY\OTHER
Scout, Unspecified

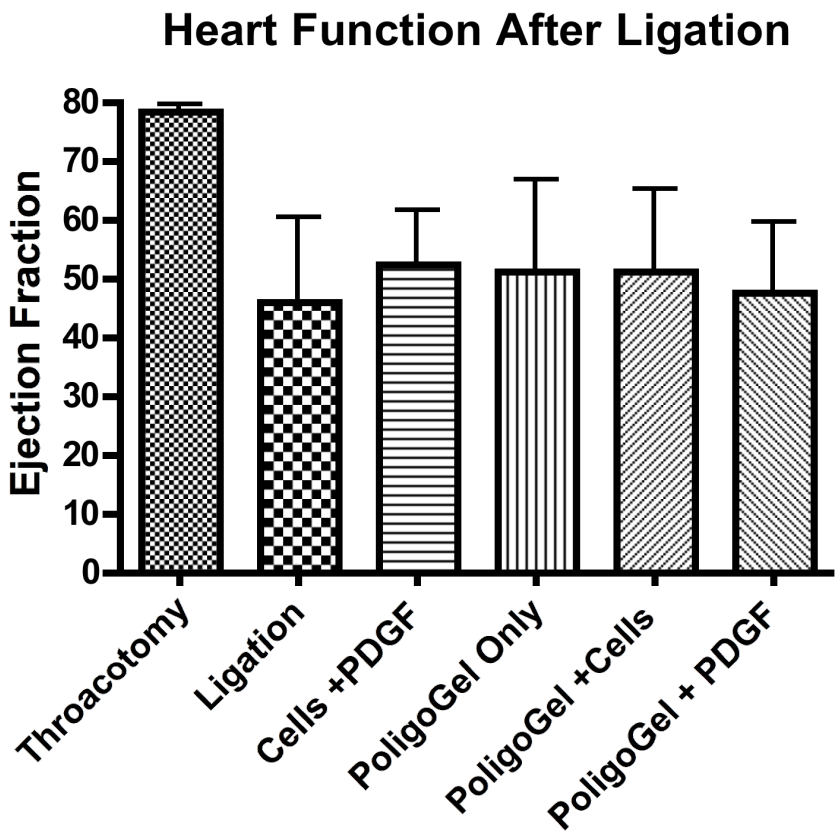


Figure 5.2 Ejection fraction results of the rat heart after ligation. While all groups were different from the nonligated control (Thoracotomy), there were no differences between the other groups. Error bars indicate the standard deviation. Comparisons were done by one-way ANOVA.

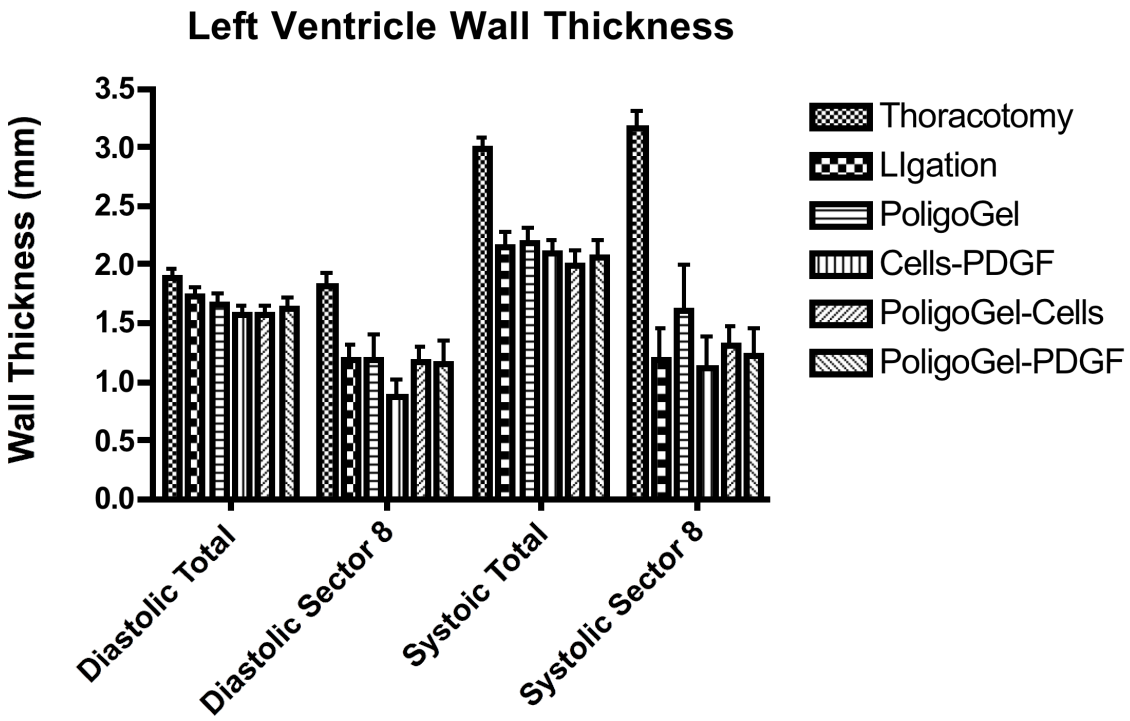


Figure 5.3 Wall thickness between treatment groups of rats. All the infarcted tissues saw decreases in thickness of the tissue, as well as loss of contractibility as indicated by limited wall thickening. Error bars are the standard deviation.

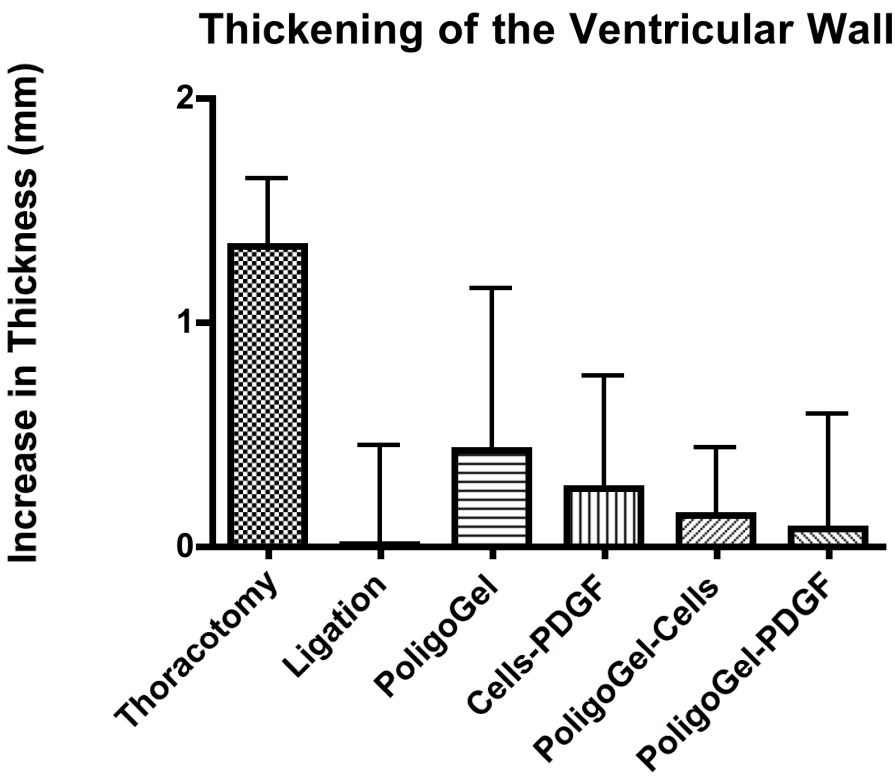


Figure 5.4 The change in the thickness of the ventricular wall for sector 8 of the heart that includes infarcted tissue. There was very little contractibility in the infarcted tissue. All of the infarcted tissue was significantly different from the control ($p<0.0001$) but there was no difference between experimental groups.

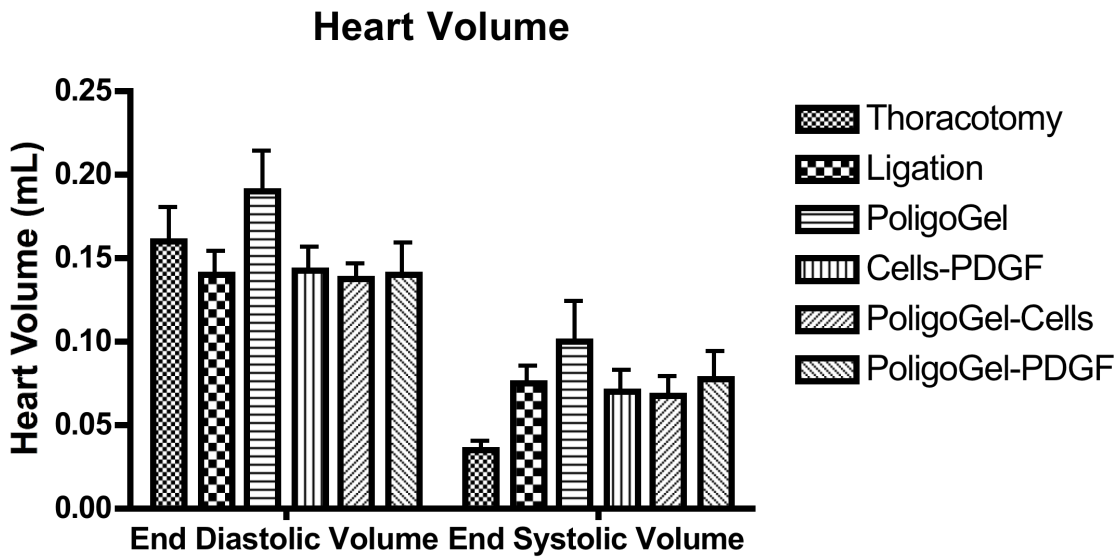


Figure 5.5 Volumes of the heart at end systole and diastole. There were no differences between the groups for either measurement. Error bars represent the standard deviation, analysis by one-way ANOVA.

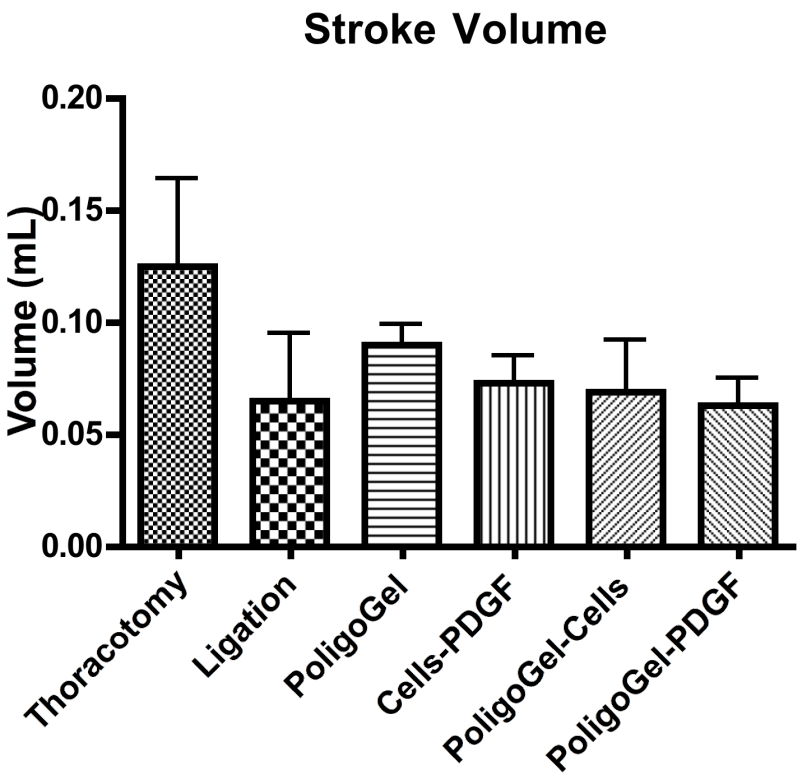


Figure 5.6 The stroke volumes for the different treatment groups. All of the groups were different from the thoracotomy control; however, there was no difference between the experimental groups. Error bars are the standard deviation; analysis was accomplished by one-way ANOVA with Bonferroni posttests.

CHAPTER 6

SYNOPSIS OF RESULTS, CONCLUSIONS, AND FUTURE PROSPECTS

6.1 Synopsis of Results and Conclusions

At the onset of this work outlined in Chapter 1, the hypothesis was three-fold. First, MSCs could effectively be used as bioreactors for the production of growth factors, and produce an angiogenic response. Second, a polymer scaffold could be found which could be used in conjunction with the MSCs without altering their intrinsic properties. The third part of the hypothesis was that the use of the MSCs in the scaffold could effectively treat the heart following a coronary ligation.

Genetically modified mesenchymal stem cells in a thermoreversible scaffold were shown to have potential for treatment for ischemic heart disease. Multiple growth factor containing plasmids were used with the MSCs and gave rise to proliferation and angiogenic responses in HUVEC, A7R5, and MSCs cell lines. Neither the growth factors, nor the polymers used for transfection, showed any adverse effects on the stem cells ability to differentiate; it also did not cause differentiation in the cells.

After the testing of multiple hydrogels, PoligoGel was found to be non-toxic and effective as a potential scaffold for the MSCs. A primary concern with the scaffold was the effect it may have on the stem cells, as the growth substrates can alter gene expression in the cell (1-3). This was not seen to be an issue with the scaffold used here. The encapsulation by PoligoGel with or without an accompanying transfection did not induce differentiation, or have any effect on the ability of MSCs to differentiate into either adipogenic fates, or osteogenic fates (4).

The combined therapy involved the study of heart function in a rat ligation model. It was expected that all groups treated with MSCs would have improved heart function over the untreated infarct group, and the addition of PDGF-C via transfection of the cells, and the use of a scaffold would yield further improvements. However, MRI results did not yield any significant difference in the treatment groups. Biodistribution indicated that the scaffold of PoligoGel was effective in retaining the cells at the heart, as measured at 24 hours. In fact, the increase was over 15-fold over cells injected without the aid of a scaffold. The single largest issue preventing statistical significance in the study was the large standard deviation in the data. Infarcts varied widely from animal to animal, making it difficult to view any effects of the different treatments. While treatment groups had higher averages than the untreated control, there was no statistical difference between any of the infarcted groups.

There are several places where changes could be made in the study that may have improved the outcome. To start with is the selection of the growth factor, PDGF-C. While this growth factor outperformed the others tested *in vitro,* its use *in vivo* did not produce any results in this study. This may have been due to inadequate gene expression, thereby failing to initiate a response of the surrounding cells. The insufficient expression could have been in the resulting concentration of PDGF-C around the wound, or the expression may not have been of sufficient duration to produce a therapeutic effect after initial exposure. The comparisons of growth factors did not extend into the animal work. Additional control groups using alternate growth factors, VEGF would be one possibility, would be needed to see if the combined therapy was ineffective as a result of the choice of the growth factor.

While PoligoGel did retain cells at the injection site at 24 hours, and has been used as a small molecule delivery vehicle *in vivo,* it may have had unanticipated and unintended consequences. While cells in the short term were at the heart, they would in most cases have still been in association with the scaffold, which was shown to limit cell attachment. It is possible that with the PoligoGel at the heart, the injected MSCs would have been unable to attach to the extracellular matrix and integrate with the heart as the polymer degraded and the gel loses integrity. The gel may also have prevented cells' movement into the infarcted region from local populations of cells, mitigating any benefit from the injected cells directly, but would not have affected the extracellular delivery of the growth factors. It is also possible that the gel

itself had adverse consequences when injected at the heart; there is no reason to suppose this alternative, but the data cannot rule it out. The spherical morphology of the cells in the gel is not believed to be an issue for several reasons. First, cells *in vitro* did not show adverse reactions to the gel while spherical; they remained viable out past 7 days, and also continued to produce proteins when transfected for over 4 days. Second, once removed from the gel, they resumed growth and proliferation.

The combined *in vivo* study had several shortcomings, some as a result of inadequate design; others were only apparent in hindsight. Large variation in the infarcts prevented the illumination of any significant differences in the groups. The large variation was at least in part due to surgical technique. Others using the same model had standard deviations about 1/3 as large as achieved here (5). A repeated measures analysis would have been a more robust method of collecting data. This would have helped to limit variation in infarct size from impairing the ability to see trends in the data. Individual animals could have had MRIs performed between days 3-7 once animals have recovered from the initial trauma of the surgery. These data would have then been compared to the data collected at the follow-up at 4 weeks postinfarct. Individual improvement or decline would not be lost in the differences between animals. Several additional control groups would have been of interest once the date came back. One of these would be the use of a different, proven growth factor; VEFG would be the likely candidate. This was neglected in large part due to the anticipated therapeutic effect of cells alone, which others have

seen (6). The use of a different gel, such as fibrin, would have also been a valid comparison. However, without improvements in the variation between animals, it is quite possible that neither of these, or any other additional control groups, would have added anything to the experiments, while raising both the financial and animal costs.

6.2 Future Work

While the present work shows promise for the treatment of heart disease, there is other work that could be done to further the understanding of the treatment. Although PDGF-CC showed consistently high results for angiogenesis *in vitro,* a different growth factor or combination of growth factors may be more effective *in vivo*. In addition to the choice of growth factor, the method of transfection may benefit from additional analysis. Using PEI, only 7-15% of the cells are transfected. Increased expression in the cells, either by refinement of the transfection, or by sorting of the cells, could also yield greater therapeutic benefit. Refinement of transfection is an ongoing process, with new polymers constantly being developed by many different labs. Sorting of transfected cells via FACS can be accomplished by labeling the plasmid or polymer prior to transfection, or by the use of a dual expression plasmid encoding green fluorescent protein, or protein marker. An additional point of consideration is the differentiation of the stem cells to a cardiac fate prior to use in the heart (7). This has not been easy to accomplish *in vitro* (8).

In addition to coronary ischemia, there are many other possible applications for transfected stem cells in a scaffold. These include stroke (9), cancer (10), diabetes (11), and neurological disorders such as Parkinson's (12, 13).

6.2.1 Stroke

Current treatments for stroke using stem cells have shown improved outcomes for patients receiving treatment (9, 14). However, these treatments have used an IV catheter for the infusion of the cells into the patent, and not a local injection. Due to the temperature-dependent gelation, it would be difficult if not impossible to inject via catheter without a gel forming prior to delivery at the desired location. It may be possible to modify PoligoGel to add a second response stimulus, such as pH, or enzymes which would delay the onset of the gel formation until after it has reached the desired location (15, 16).

6.2.2 Cancer

There are multiple genes that have been used for gene therapy treatment of cancers. Some of these genes are soluble, such as growth factor receptors (17, 18), and interleukins (19). These could be delivered in transfected stem cells and retained at the cancer site. This would be most effective after a lumpectomy, or even a biopsy. It would also be possible to treat cancer using stem cells transfected with suicide genes (20) relying on

paracrine factors to induce apoptosis in surrounding cells. Others have transfected cells with replication-competent viruses prior to treating cancer (21, 22); this can help minimize the neutralization of viral particles by the immune system before they can infect cancer cells. Any number of genes could be used with this type of approach.

6.2.3 Diabetes

Cell-based therapies for the treatment of diabetes have suffered from problems of low cell viability following transplantation (23). One proposed method of overcoming this is to use a rechargeable system, where cells and scaffold can be replaced as efficacy wanes (24). A rechargeable system such as proposed by Bae et al. coupled with modified stem cells expressing insulin driven by a glucose responsive promoter (25) could be an effective treatment for diabetes, and similar work has shown promise (11).

6.2.4 Neurological Disorders

Unlike stroke, treatment for degenerative neurological disorders may require access to the brain, such as for the implantation of electrodes for deep brain stimulation (26). This access allows for the simple injection of stem cells in PoligoGel.

6.2.5 Additional Possibilities

There is also the possibility of using the gel to deliver more than just transfected stem cells. Multiple studies have demonstrated the benefit of using multiple growth factors to achieve better outcomes (27, 28). Results have demonstrated the benefits of a multiple growth factor delivery approach. Two different populations of transfected cells could be grown and combined; or a recombinant protein could be placed in the gel. It may even be possible to place DNA / polymer complexes in the gel to be released and transfect the surrounding tissue. PoligoGel has already been used for the delivery of small molecules (US Patent 7,655,735 B2).

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